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BASIC STUDIES IN PERCUTANEOUS ABSORPTION

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BASIC STUDIES IN PERCUTANEOUS ABSORPTION

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1. INTRODUCTION

In the six month period covered by this report, two new studies have been initiated. Apparatus and techniques have been developed to measure vapor diffusion of chemicals through separated epidermis. The penetration of alcohols and amines has been investigated by this method. A second new technique developed during this period for investigating the penetration of simple agents through skin is that of electrodialysis, which has permitted the study of ionic species which do not normally penetrate skin. A new technique for use in gas chromatographic analysis of fatty acids has also been developed.

Studies on several previously reported lines of investigation have been continued. Preliminary data are presented on the hexosamine content of isolated corneum cells and a method for fractionating the "barrier lipid fraction", employing silicic acid chromatography, is described. Our studies of the role of essential fatty acids in skin have been extended with an investigation of the influence of dietary linoleic acid and linolenic acid on skin permeability. Measurements of electrical conductivity as an indication of barrier integrity have been made in *in vivo* studies on human skin. In the model membrane studies using Millipore filters, the mechanism of water transport was studied in the light of physical laws governing diffusion.

2. VAPOR DIFFUSION OF AMINES AND ALCOHOLS THROUGH EPIDERMIS

Contents

The diffusion of water vapor through skin has been studied extensively in our laboratory, and has included investigations of the effects of environmental temperature and relative humidity on the process (1, 2, 3). In order to broaden this study, the passage through dried epidermis of a number of alcohols and amines in the vapor state was measured.

In this study the permeability coefficients (diffusion rates at unit vapor pressure gradient) of straight chain alcohols and amines showed a general tendency to increase with increasing chain length above C_2. The same trend was seen with the iso-forms, but the permeability coefficients were consistently lower than those of the corresponding normal-forms. On the basis of these observations, it was concluded

that in addition to the nature of its substituent group(s), both the chain length and the extent of branching of a compound may markedly influence its rate of penetration through dry epidermis.

Further, a mathematical relation has been proposed, on purely empirical grounds, to express the permeability coefficients of the members of a particular homologous series as a function of the chain length. Using this relation it was shown that the process of amine vapor diffusion through epidermis is similar to the process of diffusion of amines in aqueous systems through whole skin.

Experimental

Apparatus - The apparatus employed for measurement of the rate of vapor diffusion through epidermis is shown diagramatically in Figure 1. In this apparatus the surface of the diffusion cell is continually swept with a fresh stream of humidity- and temperature-controlled air at a known rate, so that the concentration of diffusate at the epidermal surface exposed to the air stream remains essentially zero. Measurements were made at a flow rate of 1 liter/minute of air maintained at 0% R.H. and 25°C. Five diffusion chambers were employed in a cylinder of 0.75 liter.

Tissue - For the vapor diffusion studies, whole skin was not satisfactory, the main deterrent being the large water content of the dermis from which water is lost only very

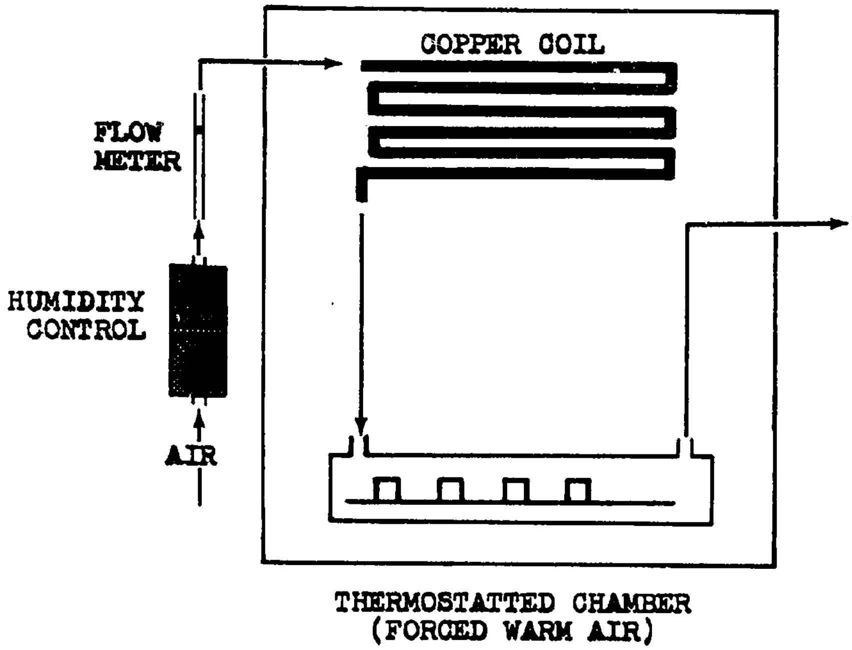

FIGURE 1. Apparatus for Vapor Diffusion Measurement.

Air under positive pressure passes into a humidity-controlling system and through a flow meter into a copper coil contained inside a thermostatically-controlled box; this allows for temperature adjustment of the air before it flows through a tube into a second closed chamber within the thermostatted box. The air sweeps over the diffusion cells placed on a rack within the smaller chamber, and the entrained vapor is carried out to the exhausting system by an exit tube.

slowly by free diffusion. Epidermis suitable for rate measurements can be readily stripped from dermis following exposure of excised skin to ammonia vapor and is best obtained from a hair-free source. Two such sources are the neonatal rat and the young adult guinea pig which has been wax-epilated (1). The epidermal membranes are prepared in large lots; they are air-dried and mounted on diffusion cell rings for later use. In the studies reported here, the rats were obtained from Hemlock Hollow Farms. Skin samples were prepared from neonatal rats averaging 8 grams in weight. The guinea pigs used were males from the Lever colony and averaged about 580 g. in body weight.

Compounds Studied - The source and an indication of purity (if known) of each of the compounds studied is given below:

Methanol: Baker Spectro Grade

Ethanol: Anhydrous, U. S. Industrial Chem.

n-Propanol: Fisher Reagent Grade, B. P. 96.9 - 97.4°C.

2-Propen-1-ol: Eastman, B. P. 95.5 - 97.0°C.

iso-Propanol: Fisher Reagent Grade, B. P. 82.3°C.

n-Butanol: Baker Reagent Grade, B. Range - 0.4°C.

iso-Butanol: Matheson, Coleman and Bell, B. P. 106 - 108°C.

sec-Butanol: Matheson, Coleman and Bell, B. P. 98 - 100°C.

tert-Butanol: Fisher Reagent Grade, B. P. 82.0 - 82.2°C.

n-Pentanol: Matheson, Coleman and Bell, Chromatoquality.

n-Propylamine: Calbiochem

iso-Propylamine: Eastman, B. P. 31 - 32°C.

n-Butylamine: Calbiochem

iso-Butylamine: Eastman, B. P. 65 - 67°C.

sec-Butylamine: Eastman, B. P. 62 - 64°C.

tert-Butylamine: Eastman, B. P. 44 - 45°C.

n-Pentylamine: Matheson, Coleman and Bell, 99.5%.

n-Hexylamine: Eastman, B. P. 129 - 130°C.

n-Heptylamine: Eastman, B. P. 153 - 155°C.

Results

A. Vapor Diffusion of Alcohols through Epidermis of the Guinea Pig and the Neonatal Rat

The permeability coefficient used to express the diffusion rates observed in this study was calculated from the relation:

$$P = \frac{w}{A \cdot t \cdot M \cdot p}$$

where: $\underline{P}$ is the permeability coefficient (μmoles·cm.$^{-2}$ hr.$^{-1}$ (cm. Hg)$^{-1}$);

$\underline{w}$ is the absolute change in weight of the diffusing material (μg.);

$\underline{A}$ is the surface area (cm.^{2});

$\underline{t}$ is the time interval during which weight loss is measured (hr.);

$\underline{M}$ is the molecular weight of the diffusing material;

$\underline{p}$ is the vapor pressure gradient across the membrane (cm. Hg), which under our conditions of measurement is taken to equal the vapor pressure of the pure liquid at the temperature of measurement.

Thickness of the membrane is not taken into account in the calculation of the permeability coefficient.

The steady-state diffusion data are given in Table I and are shown graphically in Figure 2. Data on water penetration are included for comparison.

Table IA

VAPOR DIFFUSION OF ALCOHOLS THROUGH GUINEA PIG EPIDERMIS

Compound	N[a]	Diffusion Rate	
		mg. cm.$^{-2}$ hr.$^{-1}$	µmoles cm.$^{-2}$ hr.$^{-1}$
Water	8	0.161[b]	8.95[b]
Methanol	7	0.895	28.0
Ethanol	6	0.082	1.78
n-Propanol	5	0.053	0.883
n-Butanol	5	0.048	0.649

[a]Number of separate membranes studied.

[b]Means.

Table IB

VAPOR DIFFUSION OF ALCOHOLS THROUGH GUINEA PIG EPIDERMIS

Compound	N[a]	Vapor Pressure[b]	Permeability Coefficient (P)
		cm. Hg	µmoles cm.$^{-2}$ hr.$^{-1}$ (cm. Hg)$^{-1}$
Water	8	2.38	3.76 ± .80[c]
Methanol	7	11.94	2.34 ± .24
Ethanol	6	5.18	0.344 ± .038
n-Propanol	5	2.29	0.386 ± .022
n-Butanol	5	0.850	0.763 ± .095

[a]Number of separate membranes studied.

[b]Calculated from tables in (4) for 25°C.

[c]Means and standard errors.

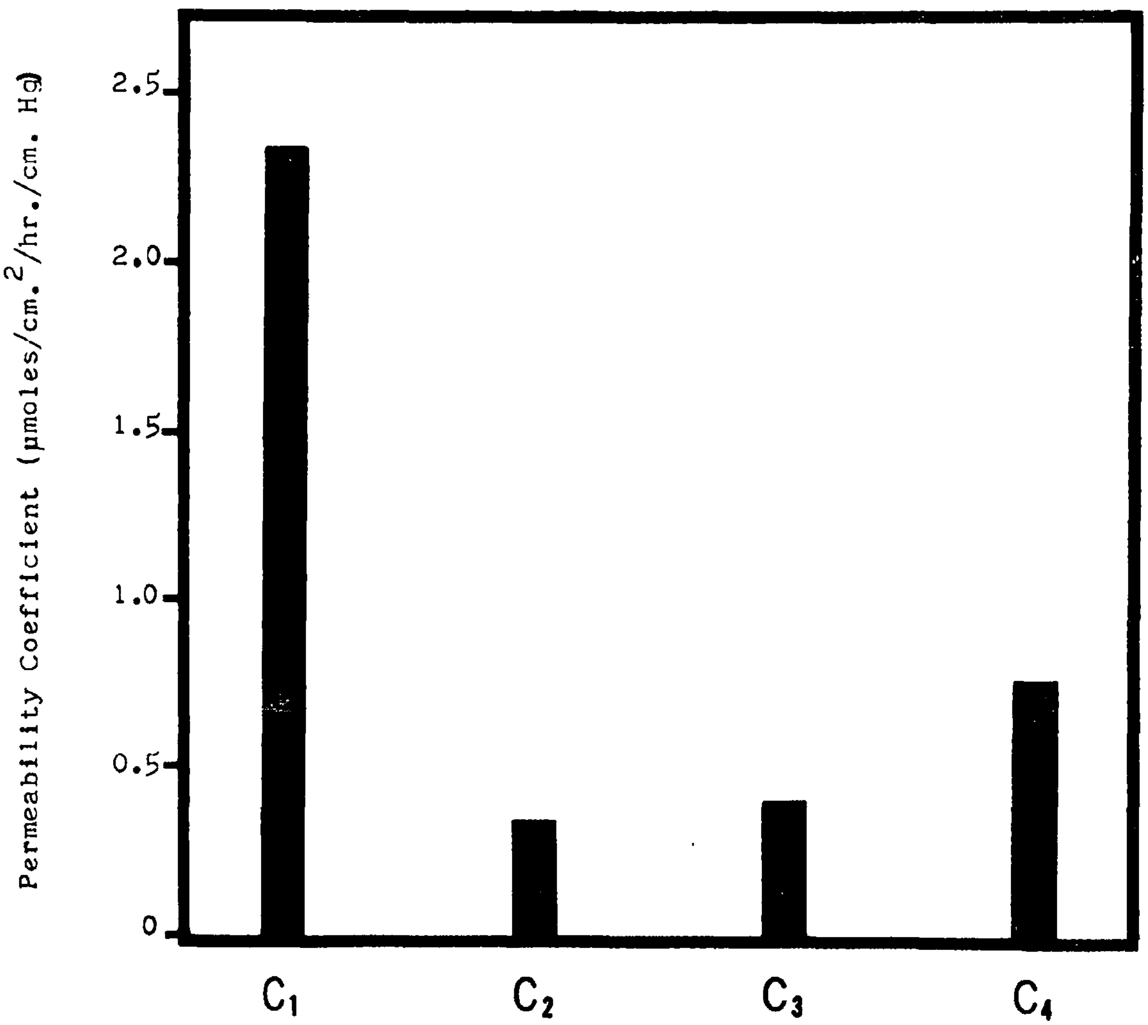

FIGURE 2. Vapor Diffusion of a Homologous Series of Primary Alcohols, C_1 - C_4, through Epidermis of the Guinea Pig.

With the exception of methanol, the diffusion rates of the alcohols are rather low. As expressed by the coefficients of permeability, the relative ease of passage of the alcohols through epidermis appears to increase with increasing chain length starting with C_2.

The data obtained for the epidermis of neonatal rats using the conditions described for guinea pig epidermis, are given in Table II and Figure 3.

Table IIA

VAPOR DIFFUSION OF ALCOHOLS THROUGH NEONATAL RAT EPIDERMIS

Compound	N[a]	Diffusion Rate	
		mg. cm.$^{-2}$ hr.$^{-1}$	µmoles cm.$^{-2}$ hr.$^{-1}$
Water	6	0.110[b]	6.12[b]
Methanol	6	0.702	21.9
Ethanol	5	0.089	1.93
n-Propanol	5	0.0254	0.423
2-Propen-1-ol	6	0.074	1.28
iso-Propanol	5	0.0148	0.246
n-Butanol	5	0.0172	0.232
iso-Butanol	5	0.0104	0.141
sec-Butanol	6	0.0168	0.227
tert-Butanol	6	0.0290	0.392
n-Pentanol	6	0.0123	0.140

[a]Number of separate membranes studied.

[b]Means.

Table IIB

VAPOR DIFFUSION OF ALCOHOLS THROUGH NEONATAL RAT EPIDERMIS

Compound	N[a]	Vapor Pressure[b] cm. Hg	Permeability Coefficient (P) µmoles cm.$^{-2}$ hr.$^{-1}$ (cm. Hg)$^{-1}$
Water	6	2.38	2.57 ± .28[c]
Methanol	6	11.94	1.84 ± .10
Ethanol	5	5.18	0.373 ± .064
n-Propanol	5	2.29	0.185 ± .034
2-Propen-1-ol	6	2.41	0.532 ± .058
iso-Propanol	5	4.73	0.052 ± .010
n-Butanol	5	0.850	0.273 ± .053
iso-Butanol	5	1.50	0.094 ± .021
sec-Butanol	6	2.06	0.110 ± .015
tert-Butanol	6	4.73	0.083 ± .006
n-Pentanol	6	0.312	0.449 ± .054

[a]Number of separate membranes studied.

[b]Calculated from tables in (4) for 25°C.

[c]Means and standard errors.

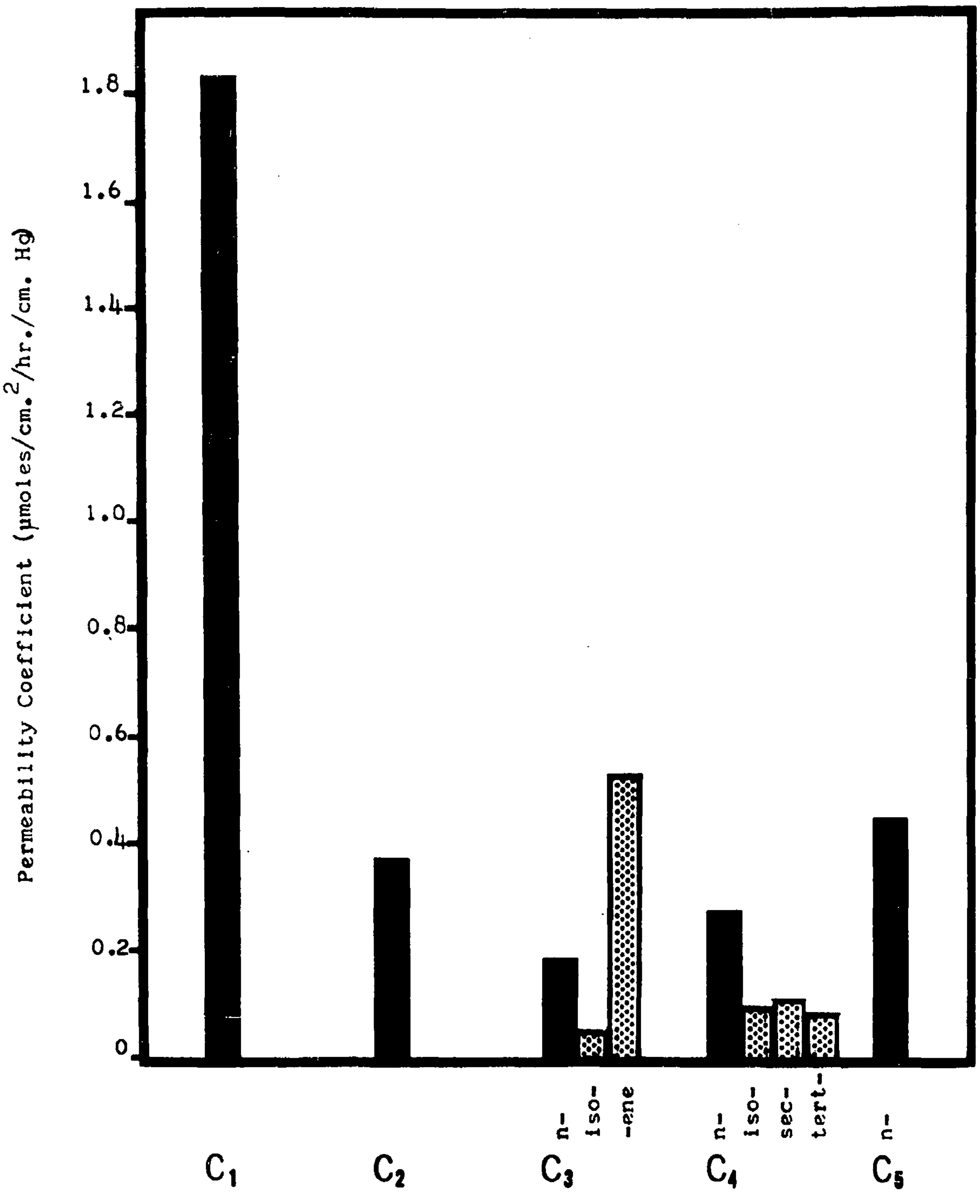

FIGURE 3. Vapor Diffusion of Straight- and Branched-Chain Alcohols, C_1 - C_5, through Epidermis of the Neonatal Rat.

The pertinent observations may be listed as follows:

1. The permeability coefficients for both water and the alcohols are lower for the neonatal rat than for the adult guinea pig.

2. The value of P is greater for water than for any of the alcohols studied.

3. For the normal saturated alcohols, P decreases as chain length proceeds from C_1 to C_3, and then gradually increases as the chain is increased through C_5. Because of the low vapor pressures of the alcohols with chain lengths greater than C_5, the study was not carried further.

4. Allyl alcohol, 2-propen-1-ol, has a surprisingly high value for P relative to its saturated C_3 counterpart, n-propanol.

5. The iso-C_3 and C_4 alcohols have values of P about one-third of those found for the normal forms of the corresponding alcohols.

6. For butanol there are only small differences in the permeabilities of the iso-, $(CH_3)_2CHCH_2OH$, sec-, $(CH_3CH_2)(CH_3)CHOH$ and tert-, $(CH_3)_3COH$, isomers.

B. Vapor Diffusion of Amines through Epidermis of the Guinea Pig and the Neonatal Rat

Amine diffusion through dry epidermis was measured using the same conditions described for the alcohol penetration measurements.

The data obtained with guinea pig epidermis are given in Table III and are shown in Figure 4.

Table IIIA

VAPOR DIFFUSION OF AMINES THROUGH GUINEA PIG EPIDERMIS

Compound	N[a]	Diffusion Rate	
		mg. cm.$^{-2}$ hr.$^{-1}$	µmoles cm.$^{-2}$ hr.$^{-1}$
Water	8	0.161[b]	8.95[b]
n-Propylamine	4	9.18	155
n-Butylamine	4	3.64	49.8
iso-Butylamine	4	2.97	40.6
n-Pentylamine	4	2.60	29.8
n-Heptylamine	4	0.692	6.01

[a]Number of separate membranes studied.

[b]Means.

Table IIIB

VAPOR DIFFUSION OF AMINES THROUGH GUINEA PIG EPIDERMIS

Compound	N[a]	Vapor Pressure[b] cm. Hg	Permeability Coefficient (P) μmoles cm.$^{-2}$ hr.$^{-1}$ (cm. Hg)$^{-1}$
Water	8	2.38	3.76 ± .80[c]
n-Propylamine	4	31.4	4.94 ± .39
n-Butylamine	4	10.13	4.92 ± .29
iso-Butylamine	4	15.42	2.63 ± .12
n-Pentylamine	4	3.54	8.42 ± .31
n-Heptylamine	4	0.362	16.6 ± 3.7

[a]Number of separate membranes studied.

[b]Calculated from tables in (4) for normal alkyl primary amines at 25°C.

[c]Means and standard errors.

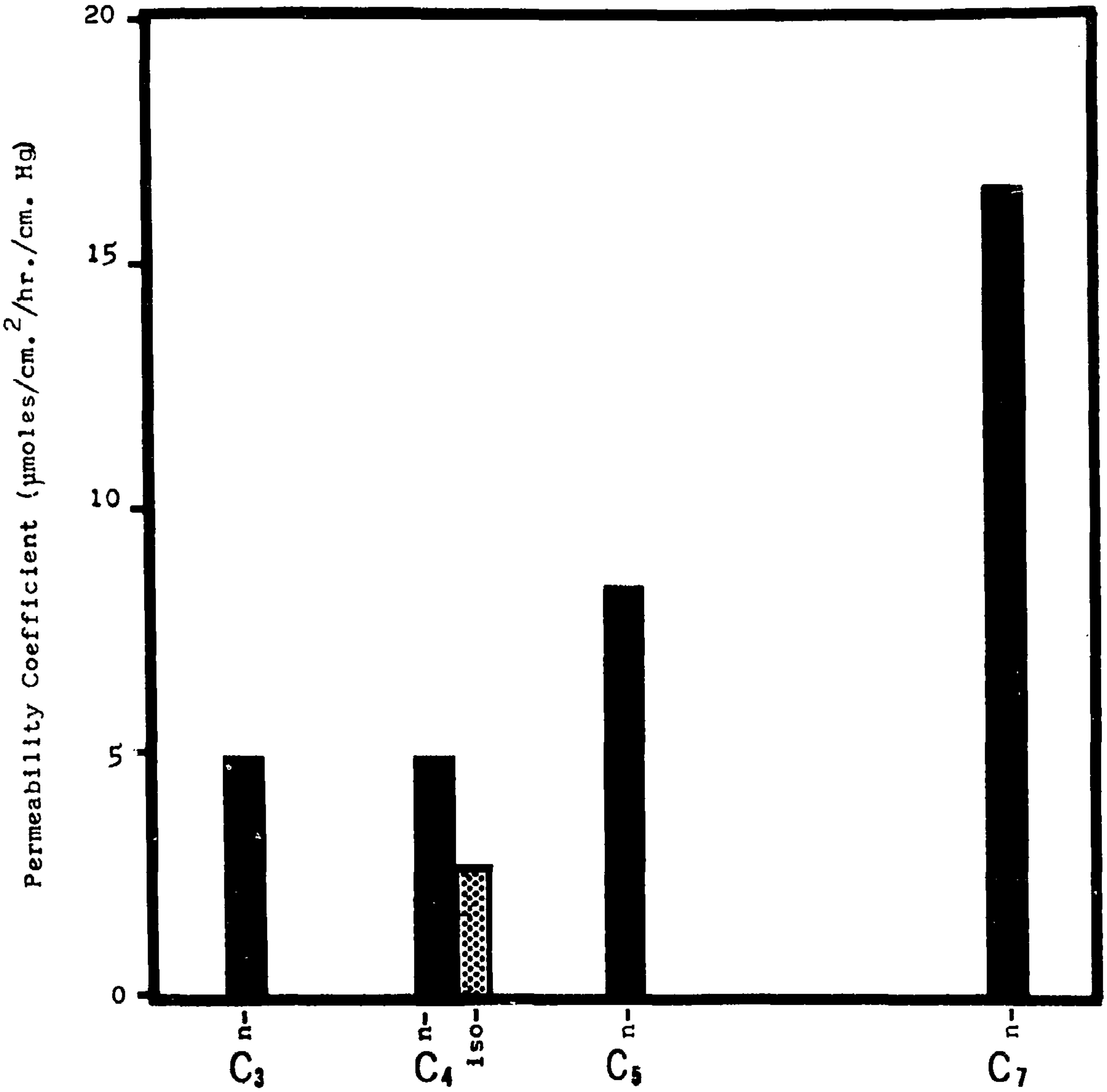

FIGURE 4. Vapor Diffusion of Straight- and Branched-Chain Amines, C_3 - C_7, through Epidermis of the Guinea Pig.

Amine penetration was very high when expressed in terms of the weight or number of μmoles passing through the epidermis; when expressed as a coefficient of permeability, the value (of P) was similar for water and the lower (C_3, C_4) amines. Since the permeability coefficients are adjusted to compare an equal number of molecular collisons with the membrane, the result observed means that an equal fraction of the collisions is "effective" for both the water and the lower amines. For this guinea pig epidermis, n-propylamine and n-butylamine showed the same value for P, while the higher amines showed an increase in P with an increase in chain length. The value of P for iso-butylamine was about one-half of that for n-butylamine.

In Table IV and Figure 5 are given the results of measurement of amine penetration through the epidermis of neonatal rats.

Table IVA

VAPOR DIFFUSION OF AMINES THROUGH NEONATAL RAT EPIDERMIS

Compound	N[a]	Diffusion Rate	
		mg. cm.$^{-2}$ hr.$^{-1}$	μmoles cm.$^{-2}$ hr.$^{-1}$
Water	6	0.110[b]	6.12[b]
n-Propylamine	4	2.31	39.1
iso-Propylamine	4	1.44	24.4
n-Butylamine	3	1.66	22.7
iso-Butylamine	3	1.24	17.0
sec-Butylamine	3	1.03	14.1
tert-Butylamine	3	0.583	7.98
n-Pentylamine	4	0.870	9.98
n-Hexylamine	4	0.560	5.53
n-Heptylamine	4	0.338	2.93

[a]Number of membranes studied.

[b]Means.

Table IVB

VAPOR DIFFUSION OF AMINES THROUGH NEONATAL RAT EPIDERMIS

Compound	N[a]	Vapor Pressure[b] cm. Hg	Permeability Coefficient (P) µmoles cm.$^{-2}$ hr.$^{-1}$ (cm. Hg)$^{-1}$
Water	6	2.38	2.57 ± .28[c]
n-Propylamine	4	31.4	1.25 ± .11
iso-Propylamine	4	55.8	0.437 ± .039
n-Butylamine	3	10.13	2.24 ± .19
iso-Butylamine	3	15.42	1.10 ± .14
sec-Butylamine	3	16.84	0.837 ± .059
tert-Butylamine	3	36.5	0.219 ± .015
n-Pentylamine	4	3.54	2.82 ± .08
n-Hexylamine	4	1.20	4.61 ± .59
n-Heptylamine	4	0.362	8.09 ± 1.93

[a]Number of membranes studied.

[b]Calculated from tables in (4) for normal alkyl primary amines at 25°C.

[c]Means and standard errors.

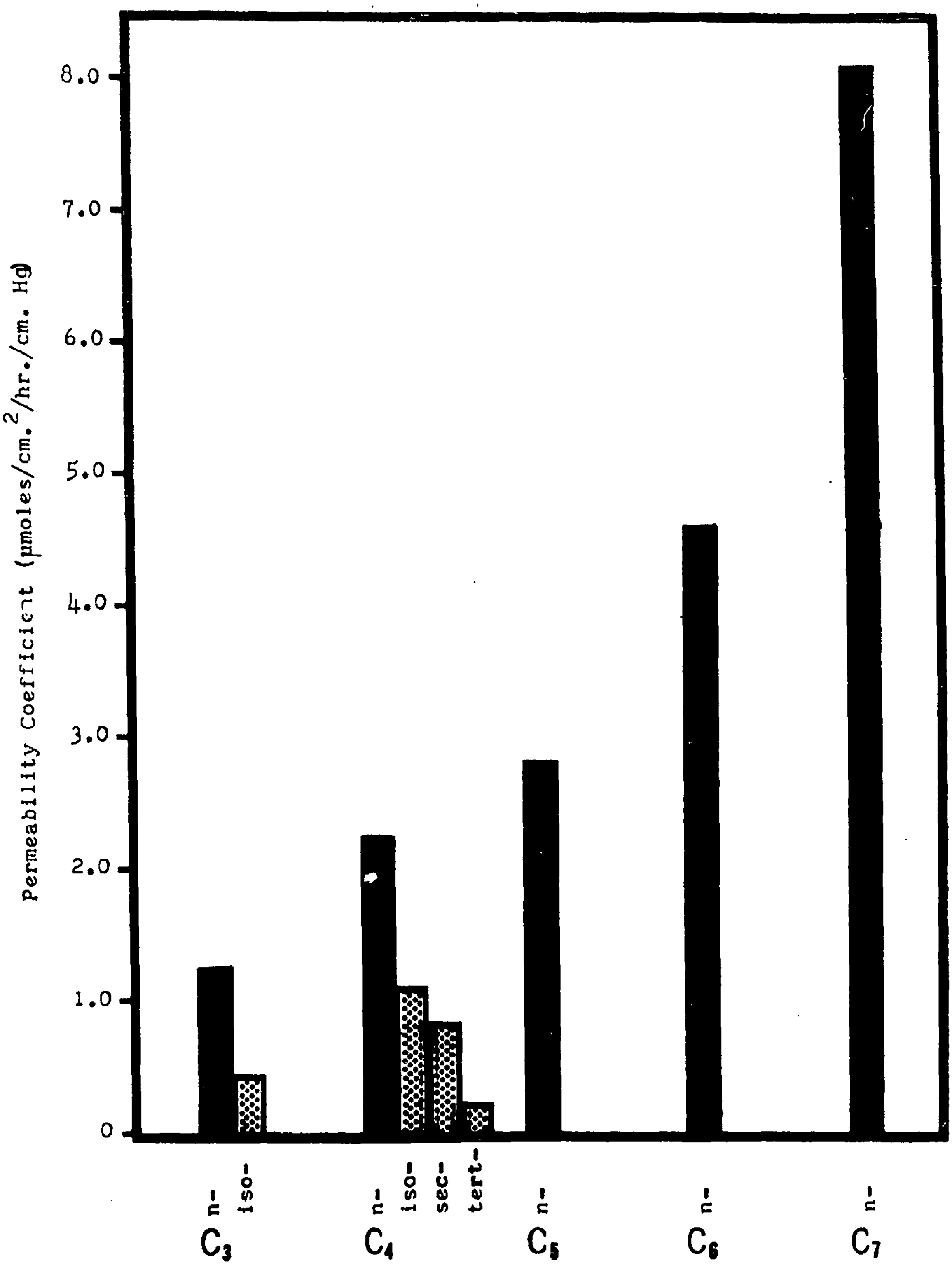

FIGURE 5. Vapor Diffusion of Straight- and Branched-Chain Amines, $C_3 - C_7$, through Epidermis of the Neonatal Rat.

The results of the foregoing experiments can be inter-related and summarized as follows:

1. As was noted in the case of the alcohols, the amines had lower permeability coefficients for neonatal rat epidermis than for adult guinea pig epidermis.

2. The general trend of increase in P values of the amines with increase in chain length that was observed with guinea pig epidermis was found to be more pronounced and also more regular with rat epidermis.

3. Iso-propylamine had a permeability coefficient about one-third of that of normal-propylamine; this relation is similar to that seen earlier when the coefficient for iso-propanol was compared with that of normal-propanol.

4. The extent of branching influenced the permeability coefficients of the C_4-amine isomers much more than those of the C_4-alcohol isomers. This was very evident when one compared the normal and tert forms of butylamine and butyl alcohol. The ratio of P for the normal and tertiary forms of butyl alcohol was 0.273/0.083, or 3.3; for butylamine, the ratio was 2.24/0.219, or 10.3. In this case, then, the effect of branching on the permeability coefficient of

butylamine was three times the effect on that of butyl alcohol.

5. As mentioned earlier in reference to guinea pig epidermis, the mass of the amines diffusing through the rat epidermis (expressed in mg. cm.$^{-2}$ hr.$^{-1}$) was in all cases much greater than the mass of water. Expressed as the coefficients of permeability, however, the value of P for water was greater than or approximately equal to the values of P for the normal and isomeric amines through chain length C_5.

C. Effect of Amine Penetration on the Water Permeability of Epidermis

One further aspect of amine penetration has been investigated - the influence of the passage of amine vapor through epidermis on the water permeability of the epidermis. This was studied by using a modified version of the water diffusion chamber. A screw was located eccentrically in the bottom of the chamber reservoir, enabling one to drain the liquid amine from the chamber after completion of a series of measurements, and to replace the amine with water. The water diffusion rate through the epidermis previously exposed to amine vapor was thus measurable without disturbing the membrane. The results of some such tests are given in Table V.

Table V

EFFECT OF EXPOSURE TO AMINE VAPOR ON THE WATER VAPOR BARRIER OF NEONATAL RAT EPIDERMIS

Amine	No. of Measurements	Water Diffusion Rate[a] mg. $cm.^{-2}$ $hr.^{-1}$
n-Propylamine	3	6.4
iso-Propylamine	1	6.0
n-Butylamine	3	5.4
iso-Butylamine	3	5.3
sec-Butylamine	3	4.9
tert-Butylamine	1	6.7
n-Pentylamine	2	5.4
n-Hexylamine	3	4.8
n-Heptylamine	1	3.5

[a]Mean values.

The data show that all of the amines were effective to some extent in destroying the water barrier of skin. Because of the incompleteness of the data, no statement will be made concerning the significance of the differences between the mean values in the table. It should be pointed out, however, that the tissues which were exposed to the more volatile shorter chain amines were thereby exposed to greater concentrations of vapor than the tissues exposed to the less volatile longer chain amines. It was reported earlier by the Lever group (1) that exposure of skin to an amine solution facilitated passage of urea through this skin.

Also, the greater the amine concentration, the higher the rate at which urea subsequently penetrated. The present system may be responding in an analogous manner.

Discussion

A trend appears to exist for the penetration of different alcohols and amines through dry epidermis. The following generalizations are suggested:

1. The permeability within the normal- or within the iso-series, increases with increasing chain length and hence, increasing distance between the polar and nonpolar ends of the molecule. This holds strictly true only for the longer chain ($> C_2$) compounds, and the strong exception of methanol must be noted.

2. The absolute penetration rates of the amines are much higher than for the corresponding alcohols, as are the permeability coefficients which express the data for unit vapor pressure gradient. For example, in neonatal rat epidermis, the value of P for n-propylamine is 1.25, vs. 0.185 for n-propanol, and for n-butylamine is 2.24, vs. 0.273 for n-butanol.

3. The straight chain compounds exhibit permeability coefficients 2 - 3 times those of the corresponding branched-chain isomers in both the alcohol and

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3. The straight chain compounds exhibit permeability coefficients 2 - 3 times those of the corresponding branched-chain isomers in both the alcohol and

amine series. The extent of branching appears to have a greater effect on the rate in the amine series than in the alcohol series. The iso-, sec-, and tert-forms of butanol, e.g., have nearly the same P value, while the corresponding isomers of butylamine show progressively decreasing values of P.

The data obtained above may be used to calculate a different permeability coefficient expressed on the basis of vapor concentration rather than vapor pressure, with the units cm./hr. This enables us to compare our data, obtained in a "dry" system, with those previously obtained in a "wet" system, using a manometric enzymatic technique for measuring amine penetration (1, 2). The values to be compared are given in Table VI.

Table VI

COMPARISON OF PERMEABILITY COEFFICIENTS FOR AMINE PENETRATION THROUGH SKIN IN "WET" AND "DRY" SYSTEMS

Compound	Vapor Diffusion[a] Perm. Coeff.[c]	Vapor Diffusion[a] Ratio[d]	Solute Diffusion[b] Perm. Coeff.[c] cm. hr.$^{-1}$ x 10^2	Solute Diffusion[b] Ratio[d]
n-Propylamine	2.31	(1.0)	1.84	(1.0)
n-Butylamine	4.16	1.8	3.17	1.7
n-Pentylamine	5.23	2.3	6.42	3.5
n-Hexylamine	8.54	3.7	6.97	3.8
n-Heptylamine	15.02	6.5	-	-
Phenethylamine	-	-	8.4	4.6

[a]"Dry" system; diffusion of amine vapor through dried epidermis.

[b]"Wet" system; diffusion of amine in aqueous solution through whole skin (1, 2).

[c]Calculated from relation: Permeability coefficient = $\frac{dn}{dt} \times \frac{1}{A\ (C_2-C_1)}$, given by Bray and White (5), where dn/dt is the rate, A the surface area, and (C_2-C_1) the concentration gradient across the membrane.

[d]The ratio of the permeability coefficients of the various amines as compared to that for propylamine.

The permeability coefficients of the amines, as measured by the "dry" system, are about 100 times greater than those found using the "wet" system. One may compare the two sets of results by using the ratio of the permeability coefficients of the various amines to that of n-propylamine (Table VI). The ratio of n-butylamine and n-hexylamine to

n-propylamine are quite close for the two systems of measurement; the pentylamine/propylamine ratios show some disparity.

If the permeability constants given in Table VI are plotted vs. the number of carbon atoms in the chain, in a semi-log plot, it can be demonstrated (Figure 6) that for both the "wet" (solute diffusion) and "dry" (vapor diffusion) systems, the log of the permeability constant is a linear function of the number of carbon atoms in the amine. That is:

$$\log P = k \cdot N_c \qquad (1)$$

where P is the permeability coefficient, k a constant equal to the slope of the line, and N_c the number of carbon atoms in the (straight) chain. Converting to Naperian logarithms,

$$\ln P = K \cdot N_c \qquad (2)$$

and

$$P = e^{KN_c} \qquad (3)$$

Similar plots of other data presented in this report have been made. In all cases where enough data were available, equation (1) was found to hold for $N_c \geq 4$.

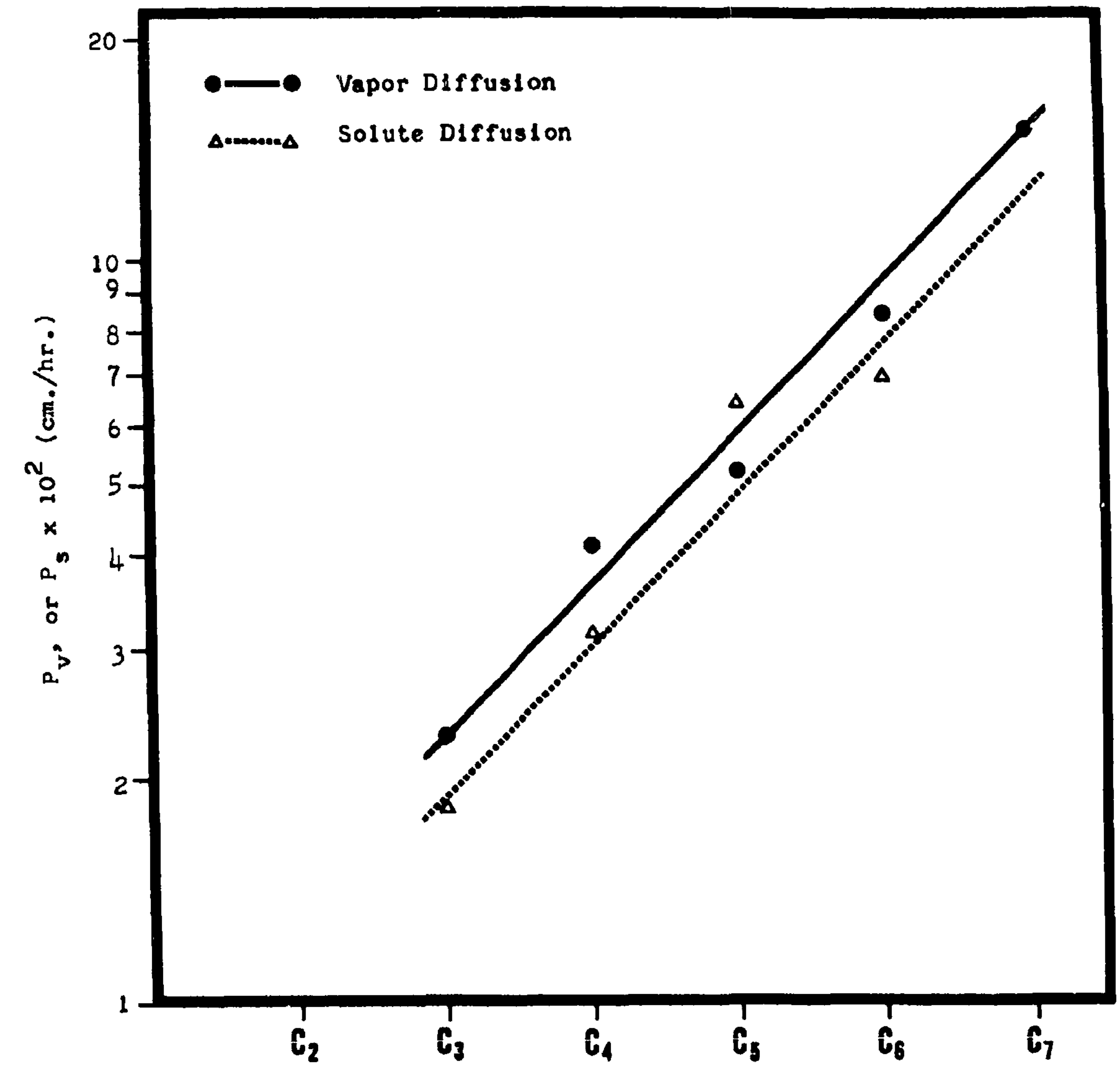

FIGURE 6. Relation of the Carbon Chain Length to the Log of the Permeability Coefficient for Vapor Diffusion (P_v) or Solute Diffusion (P_s) of n-Alkyl Amines.

to penetrate whole skin at an almost negligible rate. Using an applied potential, glutamate can be induced to penetrate the epidermal membrane at pH 7, moving toward the anode. In these studies, glutamate was assayed by the same ninhydrin procedure as was used for amine assay. Plots of ninhydrin color vs. time gave uniformly linear curves, with no lag time or "break", if conditions 1 - 4 of the procedure outlined above for the amine were met.

Glutamate was run at two concentrations, 0.25 M and 0.10 M, both dissolved in 0.001 M phosphate at pH 7.00. The results are given in Table II.

Table II

ELECTRODIALYSIS OF L-GLUTAMATE THROUGH NEONATAL RAT EPIDERMIS

Test No.	Glutamate Concentration M	Current (milliamps) Test Cell	Current (milliamps) Control Cell	Rate µmoles/coulomb
1 a	0.10	2.8	1.6	1.4
b	"	2.8		1.3
c	"	2.8		1.2
2 a	0.25	2.8	1.4	1.4
b	"	2.8		1.3
c	"	2.8		1.1
3 a	0.25	2.8	1.6	1.2
b	"	2.8		1.2
c	"	2.8		1.1
				Average 1.24

Two inferences may be drawn from the above data:

1. The concentration gradient across the membrane, at least in the range tested, is not a rate determining factor in the electrodialysis process. In the passage of charged particles through an orifice with a charge of the same sign, the particle concentration should not be a factor when the number of particles available for transport through the orifice is greater than the number of particles which can be transported by the energy available for overcoming the electrical repulsion in the system. The lowest particle concentration at which a maximal transport rate is observed per unit energy expended would then be a "saturation concentration". It is expected that this "saturation concentration" would vary directly with the total current passing through a cell.

2. The rate of transport of the glutamate anion (1.2 μmoles/coulomb) is significantly lower than that of the amine cation (2.0 μmoles/coulomb) for an equivalent amount of electricity passed through the cell. Whether the difference in rate results from a difference in the sign of the net charge, from a difference in the shape of the migrating particles, or from a difference in the polarity of the molecules, is conjectural at this time. In the first case, one would have to assume that the epidermal membrane more

strongly repulses negative charges than positive charges. In the second case, on would look to a greater frictional resistance to the passage of the glutamate, which is branched at the alpha-carbon, than to that of the straight chain amine. In the third case, absence of a charge on the terminal alkyl group of the amine would stand in contrast to the presence of a charge on the ionized distal carboxyl group of the glutamate.

In subsequent studies, the effect of treatment of the membrane in various ways on penetration rates will be studied; in addition, the effect of chain isomerism on penetration rates will be investigated, with attention to the sign of the charge, and the nature of the charged groups.

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4. FURTHER CHARACTERIZATION OF SKIN BARRIER MATERIAL

Contents

Introduction

The extraction of a lipid fraction from the stratum corneum of neonatal rats has been described (1). This fraction has been designated as the barrier lipid fraction (BLF), based on the observation that when this material was applied to a lipid free reconstituted stratum corneum membrane, the water vapor permeability of these membranes was reduced to levels found for normal skin.

Analysis of BLF by thin layer chromatography revealed at least six major components. Isolation of these components on columns of silicic acid has been undertaken in order to determine which component or combination of components is responsible for providing a barrier to water vapor diffusion.

Materials and Methods

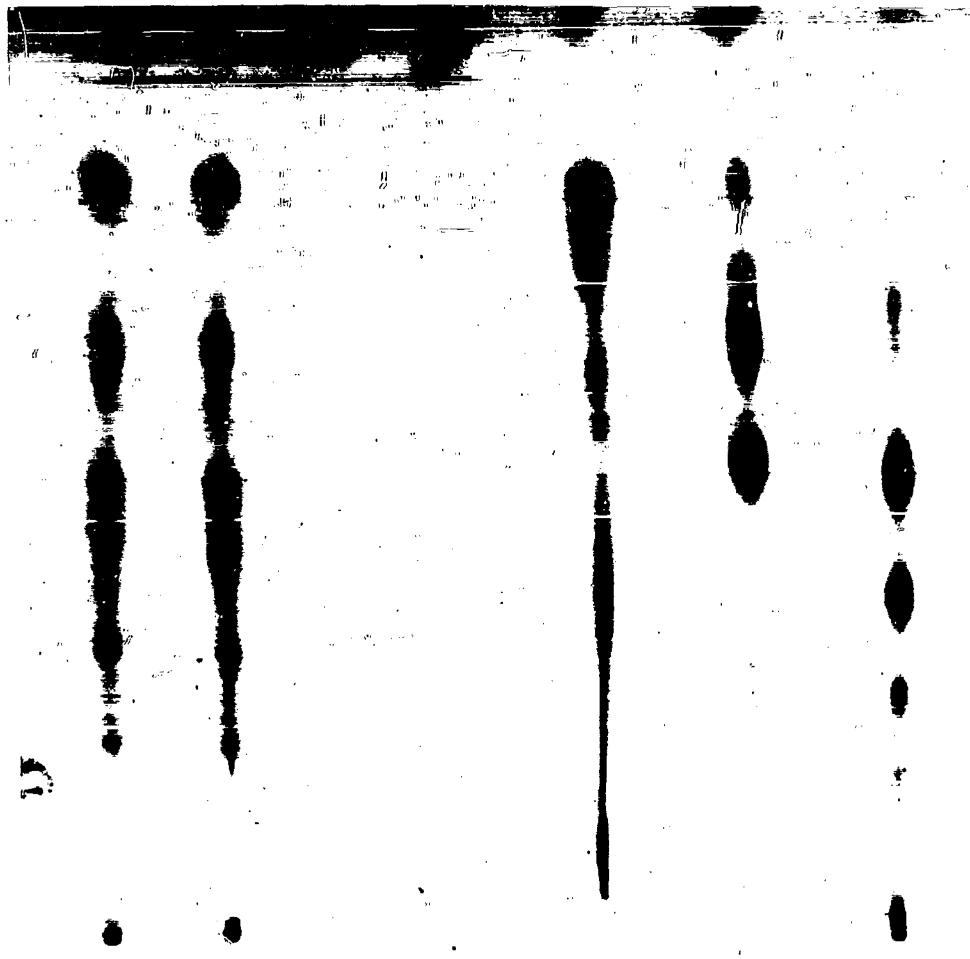
BLF was extracted from neonatal rat stratum corneum as previously described (1). Sephadex A-25, Medium was obtained through Pharmacia Fine Chemicals Inc. The

silicic acid (minus 325 mesh, chromatography grade) was obtained from Calbiochem. The organic solvents, when of "spectro" grade were used directly, reagent grade solvents were redistilled prior to use.

Sephadex columns, 1.0 x 62 cm. were prepared according to the method described by Wells and Dittmer (2). Silicic acid columns, 1.7 x 13.0 cm. were prepared following the procedure of Hirsch and Ahren (3). The lipid content of the effluent from the silicic acid columns was assayed by an acid-dichromate method (4). The amino content of 0.5 to 0.9 mg. of barrier lipid was determined using ninhydrin (5).

<u>Removal of Ninhydrin-Positive Material from BLF</u>

From 200 to 300 mg. of BLF was dissolved in a minimal volume of chloroform-methanol-water (60:30:4.5), usually about 35 ml., and was passed through a column of Sephadex at a flow rate of about 0.5 ml. per minute. The column was washed with 30 to 40 ml. of chloroform-methanol (2:1), and the combined eluants were taken to dryness in a flash evaporator. By this procedure, most of the ninhydrin positive material was removed from the BLF sample (Table I). Thin layer chromatographs of BLF before and after treatment with Sephadex appeared to be identical, indicating no major loss of lipid (Figure 1).



1 2 3 4 5 6 7

FIGURE 1. Fractionation of the Barrier Lipids on a Silicic Acid Column

Adsorbent Silica Gel H Sample Size 40 - 45 µg.

Eluant $CHCl_3$,MeOH,H_2O 75:6:0.5 Developer Chromic Acid

1. Barrier Lipid Fraction
2. " " " after Sephadex
3. Fraction I
4. " II
5. " III
6. " IV
7. " V

Table I

REMOVAL OF NINHYDRIN-POSITIVE MATERIAL
FROM BLF BY SEPHADEX

Experiment No.	% Ninhydrin Positive Material (Based on Leucine)	
	Before Sephadex	After Sephadex
1	15.4%	1.2%
2	4.8%	0.7%
3	9.7%	1.4%

Fractionation of BLF on Silicic Acid Columns

From 150 to 300 mg. of dried, Sephadex treated BLF was suspended in about 10 ml. of hexane and layered over a silicic acid column which had been equilibrated with hexane. The eluant was collected in 5 ml. fraction, and a stepwise elution program was employed. The description of the elution schedule of a typical fractionation is summarized in Table II.

The fractions eluted with each solvent were combined and spotted on a TLC plate. The separations of BLF components achieved on the silicic acid columns are shown in Figure 1. Most of the nonpolar components of BLF were eluted with hexane and hexane-benzene (5.6:1) (fractions I and II). This comprised usually about 10% of the total material placed on the column and consists probably of waxes and sterol esters. About 28% of the total material placed on the column could be eluted with benzene (fraction III)

and appears to consist primarily of sterols, as judged by the order of elution from the column. Some contamination derived from the more polar lipids was frequently observed. The remaining material was eluted with chloroform and chloroform-methanol (2:1) (fractions IV and V). The polar components comprise about 60% of the total BLF material.

Studies are in progress to evaluate the various BLF components, as separated on silicic acid columns, for their effectiveness in providing a water vapor barrier to reconstituted stratum corneum membranes.

Table II

SEPARATION OF COMPONENTS OF BLF MATERIAL ON SILICIC ACID COLUMN

Fraction	Eluting Solvent	Volume of Eluant ml.	Weight of Lipid mg.
I	Hexane	66	5.8
II	Hexane-Benzene (5.6:1)	248	23.7
III	Benzene	471	63.7
IV	$CHCl_3$	252	78.0
V	$CHCl_3$ MeOH (2:1)	67	50.0

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5. HEXOSAMINE CONTENT OF KERATINIZED CELLS OF NEONATAL RAT STRATUM CORNEUM

Contents

The isolation from stratum corneum of a lipid fraction and of separated stratum corneum cells has been described in a previous report (1). In the course of isolating the dissociated stratum corneum cells, all water soluble components and lipid constituents have been removed. Preliminary analysis of these cells indicated that they consist predominantly of insoluble protein, presumably keratin, and mucopolysaccharides bound to the insoluble proteins. Since amino sugars constitute a large part of mucopolysaccharides, an estimate of the mucopolysaccharide content of the dissociated keratinized cells can be obtained by determining the hexosamine content of the tissue.

In this report, a series of acid hydrolyses using 3, 4 and 6 N HCl, was conducted on the corneum cells of neonatal rats for time periods varying up to 24 hours and hexosamine release from the corneum was assayed. The hexosamine content of the tissue varied between 1.56 µg. and 1.8 µg. hexosamine/mg. of tissue.

Procedure

Hydrolysis Conditions

The optimum hydrolysis conditions for evaluating the hexosamine content of stratum corneum cells were determined. Samples of stratum corneum cells (40 - 50 mg.) prepared as previously described were hydrolyzed at 100°C. in sealed vials containing 6 ml. of 3, 4 and 6 N HCl for time periods varying from 2 to 24 hours. After hydrolysis, the samples were centrifuged to sediment the residue. A 5 ml. aliquot of each supernatant was placed in a 10 ml. beaker and taken to dryness under vacuum in the presence of NaOH pellets and P_2O_5. After drying, the contents of each beaker were quantitatively transferred to a test tube with four 1 ml. water washes. A 2 ml. aliquot of the combined wash was utilized in assaying the hexosamine content of the tissue hydrolysates.

Total Hexosamine Assay

Total hexosamine was assayed by the method of Cessi and Piliego (2). In this procedure, hexosamines are treated with acetyl acetone and the resulting volatile chromogens are distilled into an acid solution of p-dimethylamino-benzaldehyde. The color is read at 545 mμ.

Results

The hexosamine values obtained after a series of hydrolyses of neonatal rat stratum corneum tissue with 3 N HCl, are reported in Table I. Peak hexosamine release in this

series appears to have been reached after six hours when a value of 1.73 µg. hexosamine/mg. of tissue was observed.

Table I

HYDROLYSIS OF STRATUM CORNEUM WITH 3 N HCl AT 100°C.

Time (Hours)	Tissue Weight (mg.)	Total Hexosamine µg./mg. of Tissue
2	46.6	1.07
3	44.8	1.17
4	46.6	1.49
6	45.5	1.73
8	51.0	1.09
22	48.3	0.71

In a series of parallel experiments, the rate of destruction of D-glucosamine and D-galactosamine, under the hydrolysis conditions employed, was investigated. The results are recorded in Table II and Figure 1.

Table II

STABILITY OF STANDARD HEXOSAMINES
IN 3 N HCl AT 100°C.

Time (Hours)	D-Glucosamine µg.	D-Galactosamine µg.
0*	456	600
2	452	585
3	452	562
4	427	545
6	400	540
8	392	-
22	286	408

*Initial content.

Considerable destruction of these amino sugars occurred within the first six hours. The rate of destruction was found to be linear with respect to time, and by extrapolating the curve to zero time, a value corresponding to the original hexosamine content was obtained (see Figure 1).

Figure 2 shows the rate of hexosamine release and destruction from chondroitin sulfate and stratum corneum cells under the same conditions of hydrolysis. The mucopolysaccharide was hydrolyzed to compare the destruction of free and bound hexosamine. From the chondroitin sulfate standard, a maximum amount of hexosamine appeared to be released between three and six hours of hydrolysis, (100.3% and 99% based

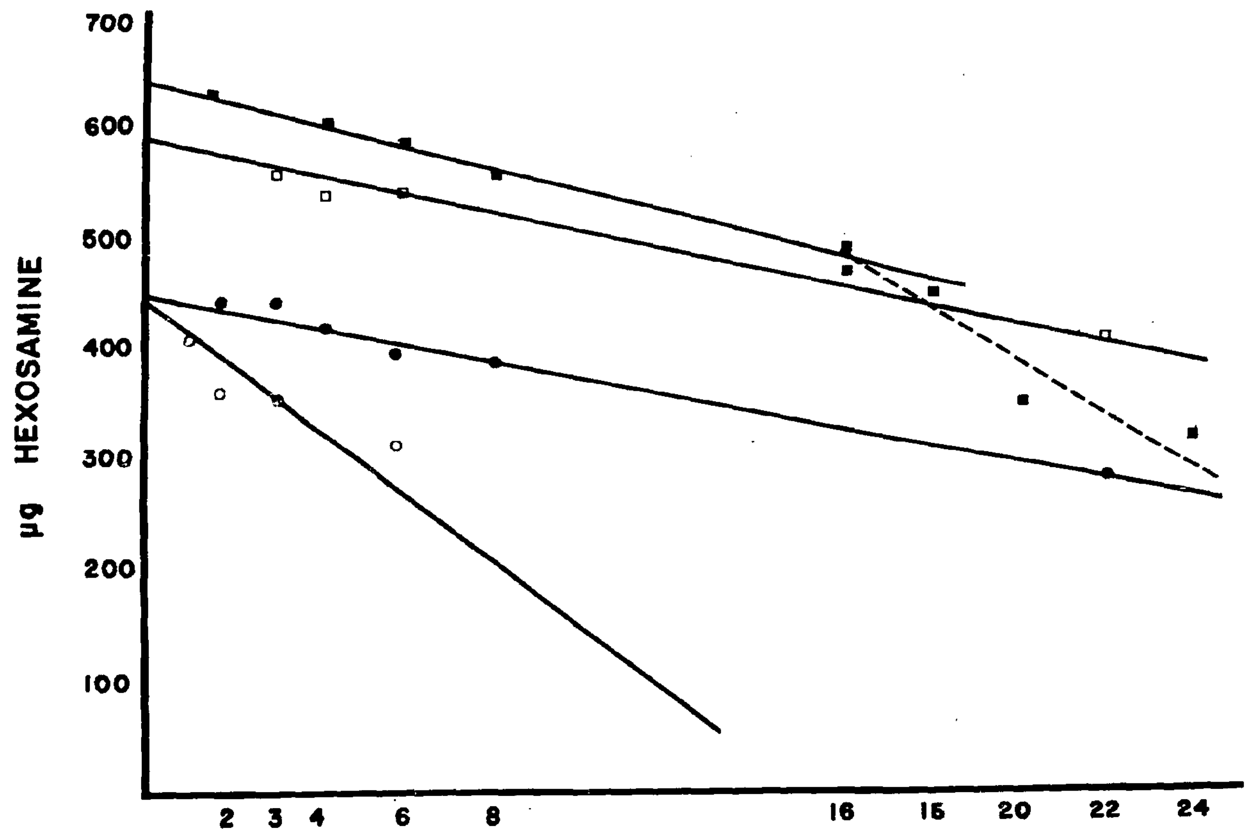

FIGURE 1. Stability of Hexosamine Standards in HCl.

■ D-Galactosamine in 4 N HCl
□ D-Galactosamine in 3 N HCl
● D-Glucosamine in 3 N HCl
○ D-Glucosamine in 6 N HCl

on a theoretical hexosamine content of 33.6%), see Table III. Extrapolating the rate of hexosamine destruction to zero time gave an estimated value of initial hexosamine content, accounting for 2.2 mg. of chondroitin sulfate versus a theoretical value of 2.0 mg.

Table III

HYDROLYSIS OF CHONDROITIN SULFATE WITH 3 N HCl AT 100°C.

Time (Hours)	Chondroitin* Sulfate mg.
0	2.0**, 2.2***
2	1.42
3	2.06
4	1.83
6	1.97
8	1.78
22	0.84

* Chondroitin Sulfate values are based on a theoretical hexosamine content of 33.6%

** Theoretical content.

*** Extrapolated value.

Assuming that a similar rate of hexosamine destruction occurred during the hydrolysis of dissociated stratum corneum cells, an initial hexosamine value was estimated for the samples by extrapolating the values of Table I to

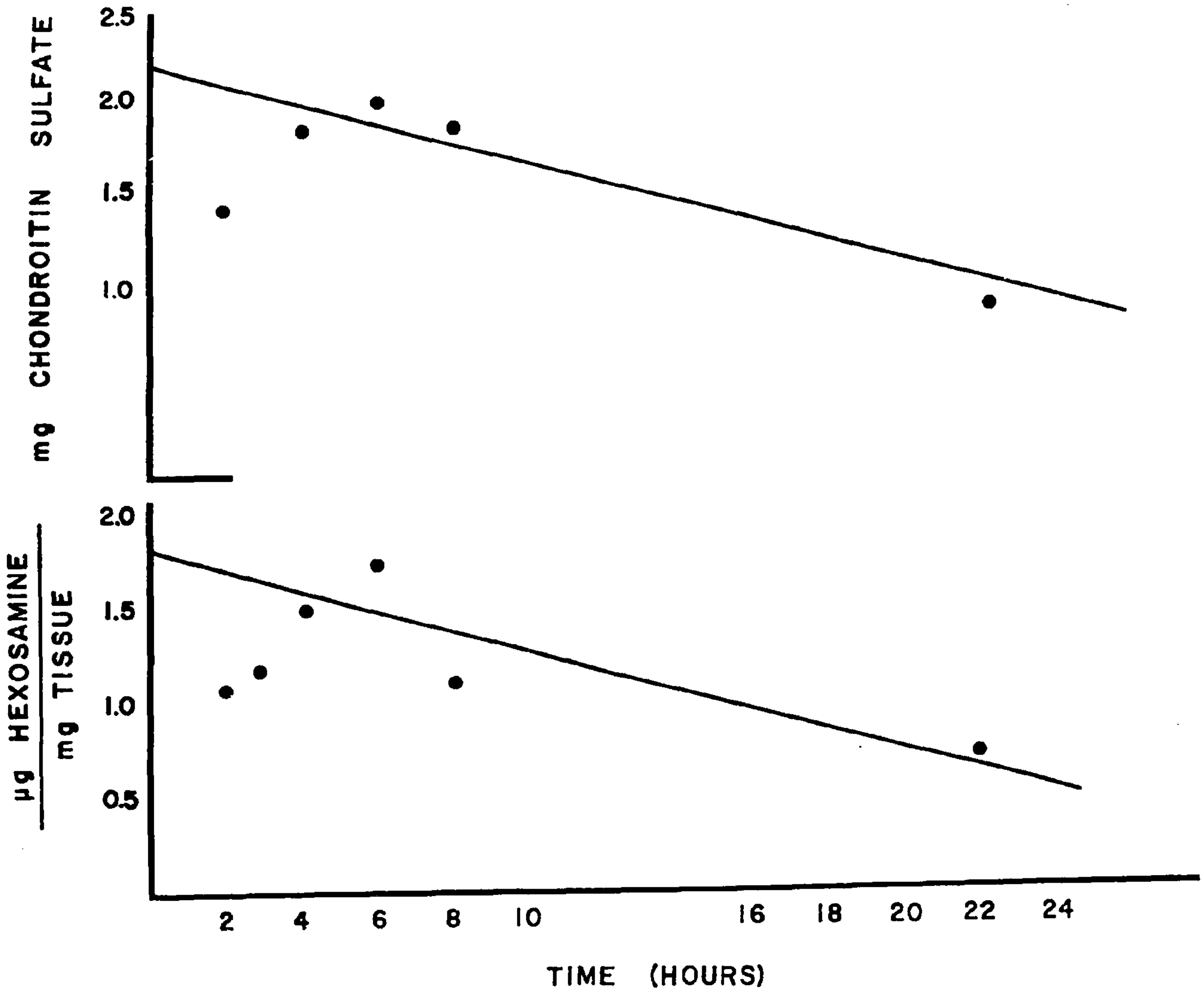

FIGURE 2. Hydrolysis of Stratum Corneum Cells and Chondroitin Sulfate in 3 N HCl.

zero time. This value was 1.8 µg. hexosamine/mg. of tissue.

In addition to the hexosamine values obtained by the method of Cessi, the 2, 4, 8 and 22 hour tissue hydrolysates of the 3 N HCl series were evaluated by the method of Dische. The values obtained by this method are not presented since a subsequent evaluation of the Dische (3) method in the presence of basic amino acids demonstrated that it was not applicable. The procedure of Boas (4) which utilized Dowex IR-50, a cationic exchange resin, was evaluated in an attempt to remove these interfering materials. The basic amino components, however, appeared to be eluted from the column with the amino sugars. No further attempt was made to develop this method.

Table IV presents the values obtained upon hydrolyzing stratum corneum cells in the presence of 4 N HCl. Extrapolating the values obtained for the first eight hours of hydrolysis to zero time, an initial hexosamine content of 1.56 µg. hexosamine/mg. of tissue was obtained. In this series of experiments, upon extending the hydrolysis time to 24 hours, a further liberation of hexosamine was observed to occur at 18 hours (see Figure 3).

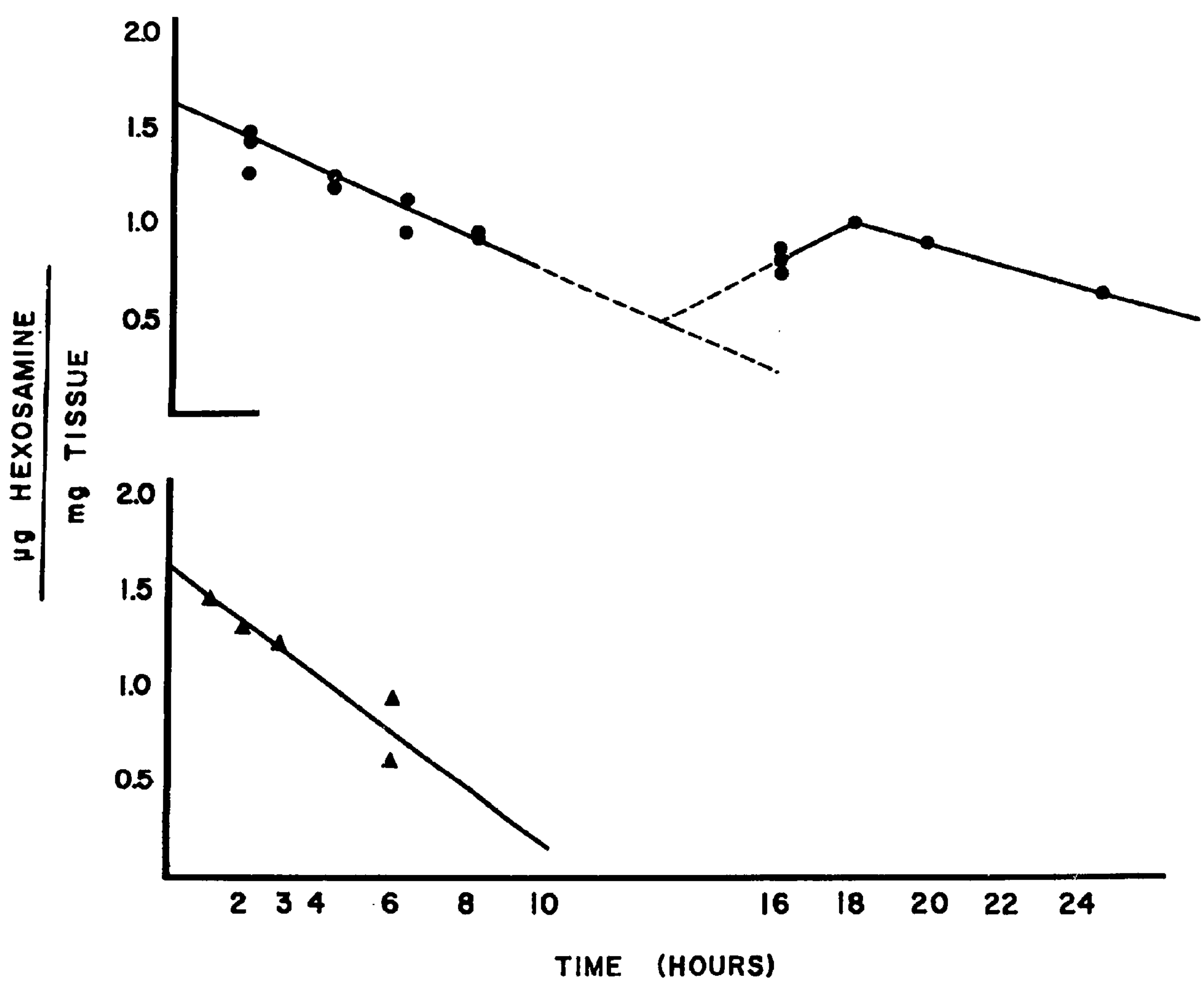

FIGURE 3. Hydrolysis of Stratum Corneum Cells in the Presence of 4 and 6 N HCl.

● 4 N HCl
▲ 6 N HCl

Table IV

HYDROLYSIS OF STRATUM CORNEUM
WITH 4 N HCl AT 100°C.

Time (Hours)	Tissue Weight (mg.)	Total Hexosamine µg./mg. of Tissue
2	42.6	1.42
2	47.3	1.39
2	42.7	1.24
4	49.3	1.20
4	42.8	1.22
6	44.2	0.94
6	41.6	1.11
8	43.5	0.89
8	45.9	0.89
16	38.9	0.73
16	47.3	0.75
16	43.2	0.81
18	38.2	0.96
20	42.2	0.83
24	42.9	0.63

In another series of experiments, the amount of hexosamine released from stratum corneum cells hydrolyzed in 6 N HCl was investigated and is reported in Table V. Under these hydrolysis conditions, an extrapolated value of 1.6 µg. hexosamine/mg. of tissue was obtained.

Table V

HYDROLYSIS OF STRATUM CORNEUM
WITH 6 N HCl AT 100°C.

Time (Hours)	Tissue Weight (mg.)	Total Hexosamine µg./mg. of Tissue
1	50.5	1.48
2	50.8	1.30
3	50.2	1.20
6	51.5	0.90
6	100.1	0.57

The extrapolated values obtained from the three series of tissue hydrolyses are presented in Table VI.

Table VI

THE HEXOSAMINE CONTENT OF DISSOCIATED
STRATUM CORNEUM CELLS

HCl Concentration	Total Heoxsamine µg./mg. of Tissue
3 N	1.8
4 N	1.56
6 N	1.6

Discussion

The hexosamine value determined for three series of acid hydrolyses conducted on dissociated cells of neonatal rat

stratum corneum indicated the hexosamine content of the cells to be approximately 0.2% (between 1.56 µg. to 1.8 µg. hexosamine/mg. of tissue). The corneum cells were prepared from epidermis by a trypsin urea digestion of the tissue, followed by water and chloroform/methanol extractions of the corneum sheets. Any hexosamine remaining with the cells would appear to be in a form which is intimately associated with keratin, and would not necessarily reflect the total mucopolysaccharide content of the intact stratum corenum. Therefore, these results can not be compared with hexosamine levels reported for whole epidermis (5) (6).

In the prolonged hydrolysis of corneum cells with 4 N HCl, an additional release of hexosamine was observed after 16 hours of hydrolysis. The hexosamine released after 16 hours of hydrolysis may reflect the release of a more resistant material. Since the occurrence of a second hexosamine release was not observed in the other hydrolysis series, the possibility of an artifact cannot be excluded. Further work is necessary to confirm this observation.

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6. EFFECTS OF SUPPLEMENTATION WITH LINOLEATE OR LINOLENATE ON THE SKIN PERMEABILITY AND GROWTH RATE OF EFA-DEFICIENT RATS

Contents

In an earlier study (1) EFA-deficient rats were supplemented with essential fatty acid (as safflower oil); the improvement in the barrier properties of skin was followed by *in vivo* electrical conductivity measurements and by estimations of the water diffusion rate of excised skin. Good correspondence was found between the changes in skin permeability as measured by the *in vivo* and *in vitro* methods.

In the current study, the activity of linolenic acid and of linoleic acid in enhancing the barrier properties of EFA-deficient skin was studied. Changes in body weight and skin permeability were followed during five weeks of supplementation of the EFA-deficient rats. Linolenic acid, fed at a final level of 100 mg. per rat per day, enabled resumption of growth but did not change the skin permeability (electrical conductivity, water diffusion rate). Feeding of linoleic acid (as safflower oil), at an estimated level of 18 mg. per rat per day, resulted in a

reduction of skin permeability after one week, and after one month the permeability was equal to that of the control. At a level of 7 mg. of linoleic acid per day, skin permeability was not significantly decreased until the third week of supplementation and skin conductivity never attained the low value of the control.

Experimental Procedure and Materials

Twenty male rats (Hemlock Hollow Farms, Wayne, N. J.) were maintained on a fat-free diet, to which was added hardened coconut oil at a level of 10%, with food and water provided *ad libitum*. Another six rats served as controls, being fed the same diet supplemented with 0.5% safflower oil (estimated to be 72% by weight linoleic acid). After 9½ weeks, the animals were subdivided into five groups (one control, four deficient), and supplementation initiated according to the scheme outlined on page 75.

Initially each rat was provided with 10.0 g. of diet per day; since this level was insufficient for maintenance of weight, the *per diem* level was increased to 15.0 g. per rat. The diet was restricted in quantity so that there would be complete consumption of the provided amount - the caloric intake was therefore identical for each of the rats and the amount of supplement ingested was known.

Group	History	Designation	Treatment
I	EFA-Deficient	Deficient	EFA-deficient diet.
II	" "	Linolenic Supplemented	EFA-deficient diet plus 35 mg. linolenic acid per rat per day; 100 mg. linolenic acid per day from 22nd day on.
III	" "	Linoleic Supplemented (7 mg.)	EFA-deficient diet plus 10 mg. safflower oil (estimated 7 mg. linoleic acid) per rat per day.
IV	" "	Linoleic Supplemented (18 mg.)	EFA-deficient diet plus 25 mg. safflower oil (estimated 18 mg. linoleic acid) per rat per day.
V	EFA-Control	Control	EFA-deficient diet plus 50 mg. safflower oil (estimated 35 mg. linoleic acid) per rat per day.

The linolenic acid used was obtained from Nutritional Biochemical Corp. (Cleveland, Ohio). No impurity was detectable at a level greater than 1% when analyzed by gas-liquid chromatography.

Electrical conductivity measurements of skin were made about every second day, starting with the first day of the experiment. At the conclusion of the study, the animals were sacrificed and the water diffusion rate of the excised skin measured.

Results

The terminal data are summarized in Table I .

Table I

SKIN PERMEABILITY AND BODY WEIGHT CHANGES OF SUPPLEMENTED RATS

Group	No. of Rats	I[a] µamp.· V^{-1}	DR[b] mg. $cm.^{-2}$ $hr.^{-1}$	Δ BW[c] g.
I EFA-Deficient	5	11.3 ± 0.7[d]	0.94 ± .09	-4
II Linolenic (35 - 100 mg.)	5	9.4 ± 0.4	0.73 ± .12	+28
III Linoleic (7 mg.)	4	4.1 ± 0.7	0.44 ± .16	+42
IV Linoleic (18 mg.)	6	1.41± 0.13	0.14 ± .01	+48
V Control	6	0.93± 0.05	0.094 ± .017	+19

[a]Electrical conductivity.

[b]Water diffusion rate.

[c]Change in body weight from the beginning to the end of the experiment.

[d]Means and standard errors.

Skin permeability (I, DR) of the EFA-deficient rats was not significantly affected by linolenic acid supplementation, whereas the effect on body weight gain was quite pronounced. Linoleate supplementation, even at an estimated level of only 7 mg./day/rat, produced a significant decrease in the permeability of skin, accompanied by a greater weight gain than was seen with linolenate supplied at a much higher level. The water permeability of the skin of two of the four rats supplied with linoleate at the 7 mg. level was in the control range, while the conductivity of all four rats was still elevated. Inspection of our previous data (1) has indicated that a similar situation existed in our earlier study of repletion - i.e. water diffusion rate returned to the "normal" level more quickly than did electrical conductivity.

The permeability and weight data obtained during the course of the study are presented graphically in Figures 1 and 2.

The following may be noted for the various groups:

Group I: The skin conductivity of the unsupplemented EFA-deficient animals showed a quasi-cyclic variation with a period of about 30 days, and a mean value of about 11.8 μamp./volt.

Group II: The linolenic acid-supplemented rats showed a similar cyclic variation in skin conductivity, slightly out of phase with the unsupplemented group (I), tending to give

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FIGURE 1. Effect of Supplementation of EFA-Deficient Rats with Linolenate or Linoleate on Electrical Conductivity of Skin.

On the fifth day of the experiment, the amount of diet supplied was increased from 10 g. to 15 g. per rat per day, for all groups. On the 22nd day, the amount of linolenic acid supplied to Group II was increased from 35 mg. to 100 mg. per rat per day.

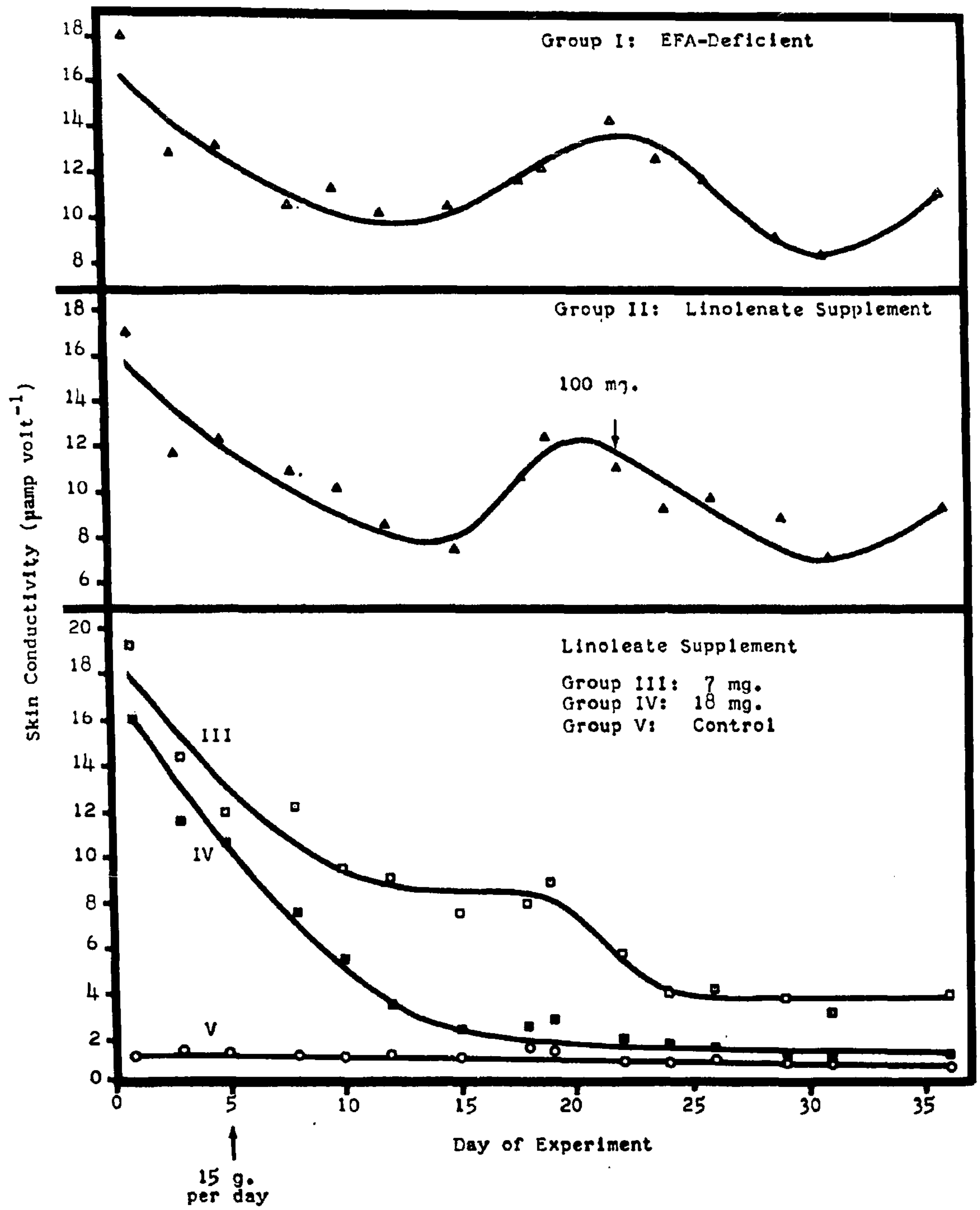
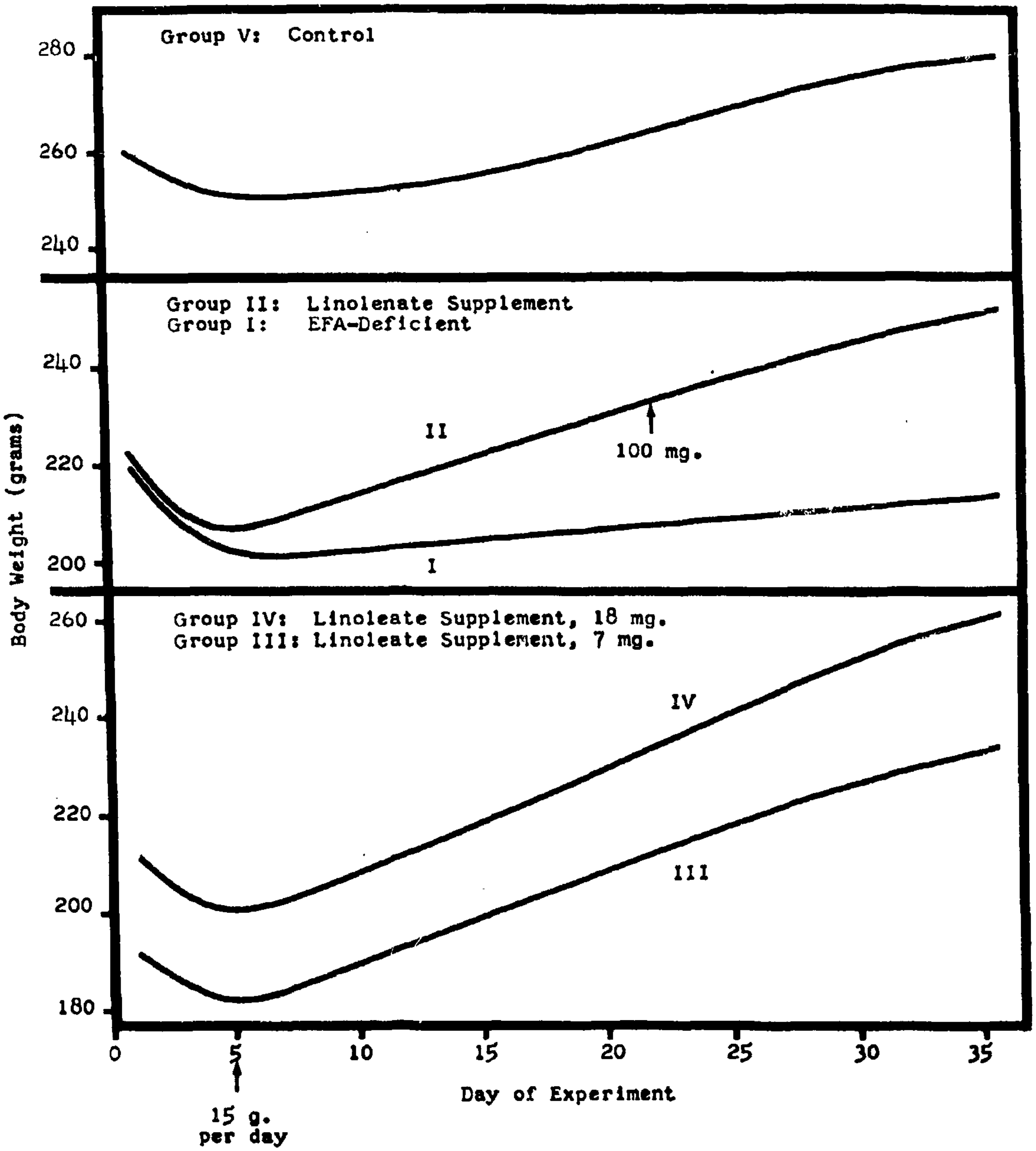

Group I: EFA-Deficient
Group II: Linolenate Supplement
100 mg.
Linoleate Supplement
Group III: 7 mg.
Group IV: 18 mg.
Group V: Control
III
IV
V
Skin Conductivity (µamp volt^{-1})
Day of Experiment
15 g.
per day

FIGURE 2. Effect of Supplementation of EFA-Deficient Rats with Linolenate or Linoleate on Body Weight.

Details of supplementation are given in legend for Figure 1 and in text.



Group V: Control
280
260
240
Group II: Linolenate Supplement
Group I: EFA-Deficient
240
220
200
II
100 mg.
I
Group IV: Linoleate Supplement, 18 mg.
Group III: Linoleate Supplement, 7 mg.
260
240
220
200
180
IV
III
Body Weight (grams)
0
5
10
15
20
25
30
35
Day of Experiment
15 g.
per day

the false impression of significant decreases in permeability as a result of linolenic acid feeding.

<u>Group III</u>: The skin permeability of the rats supplemented with a safflower oil-equivalence of 7 mg. per day of linoleate showed only a slow response to supplementation. The skin conductivity values seemed to be in phase with those for the linolenate-supplemented rats, but the values gradually decreased, finally leveling out at a value significantly above the controls.

<u>Group IV</u>: Skin conductivity of the rats supplemented with safflower oil equivalent to 18 mg. per day of linolenic acid decreased rapidly through the 14<u>th</u> day of supplementation, followed by a slow, gradual decline to the control level. In our previous study with supplementation at a higher level of safflower oil, a somewhat similar situation was observed.

<u>Group V</u>: The control animals showed no appreciable variation in skin conductivity during the experimental period, remaining at a low level near 1.2 μamp/volt.

Discussion

A study of the effects of dietary supplementation of EFA-deficient rats with various unsaturated fatty acids was

carried out by Mohrhauer and Holman (2). With respect to weight gain, the order arachidonate $>$ linoleate $>$ linolenate was observed. With respect to remission of dermal deficiency symptoms, arachidonate was more effective than linoleate, with linolenate running a very poor third. In their work, Mohrhauer and Holman related the changes in weight and in dermal symptoms to the fatty acid level as a percent of the caloric intake, not to the absolute weight of fatty acid provided per rat per day, as we have done. It also appears that their diets were fed ad libitum, while in our study, the dietary intake was restricted to an amount that would be completely consumed by all of the animals. In spite of these differences, good agreement was found between the studies concerning the effects of linoleic and linolenic acids on EFA-deficient rats.

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7. A METHOD FOR SEPARATING SATURATED FATTY ACID ESTERS FROM UNSATURATED ESTERS FOR GAS CHROMATOGRAPHIC ANALYSIS

Contents

In a previous communication (1), we have described our investigation of the fatty acid composition of several epidermal lipid fractions. Included were samples obtained from control rats and from rats whose epidermal barrier properties had been altered by maintaining the animals on a diet deficient in essential fatty acids. These lipids were analyzed by a combination of thin layer and gas chromatographic techniques. The gas chromatograms of the fatty acid esters invariably contained incompletely resolved peaks (1).

In order to evaluate these data, it was essential to establish which of the components were saturated and which were unsaturated. A widely recommended procedure for differentiating between saturated and unsaturated fatty acids is bromination of the mixture prior to gas chromatography to eliminate peaks due to the unsaturated components (2). In our hands, the application of this method to the analysis of fatty acids derived from skin lipids had only limited success. Although the peaks due

to unsaturated fatty acids were substantially reduced, they were never entirely eliminated and new peaks, as yet unidentified, were observed.

We therefore developed a procedure for quantitatively separating the saturated fatty acid esters from the unsaturated esters by bromination on a thin layer chromatographic plate. After isolation, the saturated esters are analyzed by gas chromatography. Comparison of the chromatogram of this fraction with a chromatogram of the complete sample permits direct identification and measurement of the saturated components. The unsaturated components are calculated by difference.

Materials and Methods

Reagents

Methyl stearate and methyl oleate were obtained from Applied Science Laboratories, Inc., State College, Pa. The methyl stearate was found to be better than 99% pure by gas chromatography. Methyl 9,10-dibromostearate was prepared by bromination of purified methyl oleate. The dibromo ester was purified by thin layer chromatography to remove traces of methyl oleate.

Thin Layer Chromatography - Desaga-Brinkman apparatus was used. The stationary phase was a 250 µ layer of Silica Gel G (Brinkman Instruments, Inc.). The eluant used to separate esters of saturated fatty acids from unsaturated esters was

hexane-ether 19:1 containing 10 drops of bromine per 100 ml.

X-Ray Fluorescence - A Norelco X-ray Spectrograph was used to detect bromine in fractions separated by thin layer chromatography. As little as 2 μg. of bromine, as methyl 9,10-dibromostearate adsorbed on 30 mg. of Silica Gel G, could be detected.

Gas Chromatography - A Barber-Colman Model 10 fitted with an 8 ft. column and a tritium ionization detector was used. For most work, the stationary phase was 20% ethylene glycol glutarate resin on 80 - 100 mesh Chromosorb W. Column temperature was 180°C., injection 300+°C., detector 205°C. Argon carrier gas pressure was 40 psig. For a nonpolar phase, 15% Apiezon L on 80 - 100 mesh Chromosorb W was used in a 10 ft. column maintained at 225°C. Column efficiencies as calculated by the formula

$$\text{number of theoretical plates} = 16 \left(\frac{\text{retention volume}}{\text{peak width}}\right)^2$$

were 1000 theoretical plates for the polyester column and 5500 theoretical plates for the Apiezon L column.

Results

Table I shows the results obtained when a mixture containing 20.2% methyl stearate and 79.8% methyl 9,10-dibromostearate was chromatographed on the ethylene glycol glutarate column.

In addition to the large peak due to methyl stearate (I), four other major peaks were seen. The combined area of these four peaks was 92% of the area of peak I. It seemed unlikely that any of these peaks was due to methyl 9,10-dibromostearate, which would be expected to have a much longer retention time on the basis of its high molecular weight and low volatility. The carbon number (3) of peak II was almost identical to that obtained on the same column for methyl oleate.

Table I

CARBON NUMBERS AND PERCENT PEAK AREA OF COMPONENTS OBSERVED AFTER CHROMATOGRAPHY OF METHYL 9,10-DIBROMOSTEARATE AND METHYL STEARATE ON ETHYLENE GLYCOL GLUTARATE

Peak	Carbon Number[a]	Percent Peak Area[b]
I	18.0	52
II	18.4	29
III	18.9	6
IV	20.2	6
V	20.8	7

[a]Carbon numbers were calculated by the method of Woodford and van Gent (3). The carbon number of methyl oleate on this column was 18.35 ± 0.05.

[b]Injected mixture contained 20.2% methyl stearate and 79.8% methyl 9,10-dibromostearate.

Figure 1 shows a chromatogram of methyl 9,10-dibromostearate obtained on the Apiezon L column. Again four major peaks were present, one of which, peak A, had a carbon number of 17.8, close to that of methyl oleate on an Apiezon L column, 17.7.

A sample of pure methyl 9,10-dibromostearate was injected into the ethylene glycol glutarate column and the effluent was collected by condensing the argon carrier gas with liquid nitrogen. Samples of the original material, the collected effluent, and an effluent blank were analyzed by thin layer chromatography on Silica Gel G using hexane-ether 24:1 (Figure 2). The methyl 9,10-dibromostearate migrated as a single component. After the sample had passed through the gas chromatographic column, it consisted of a mixture, one component of which (A) had the same relative migration rate on thin layer chromatography as methyl oleate. Methyl 9,10-dibromostearate would not be expected to be eluted from the column during the collection period.

A second larger sample (160 μg.) of the material eluted from the gas chromatographic column after injection of methyl 9,10-dibromostearate was separated by thin layer chromatography. The three major components (labelled A, B and C in Figure 2) were scraped off the plate and analyzed by X-ray fluorescence. All three fractions were found to be free of bromine. Under the conditions

FIGURE 1. Gas chromatogram obtained from Apiezon L column after injection of methyl 9,10-dibromostearate. Peaks S_1 and S_2 are due to methyl myristate and methyl palmitate, added as standards for carbon number calculations (3). The carbon numbers calculated for the other peaks were A = 17.8, B = 18.2, C = 18.4, D = 18.7. The portion of the curve between the vertical arrows was recorded at an attenuation of 2X.

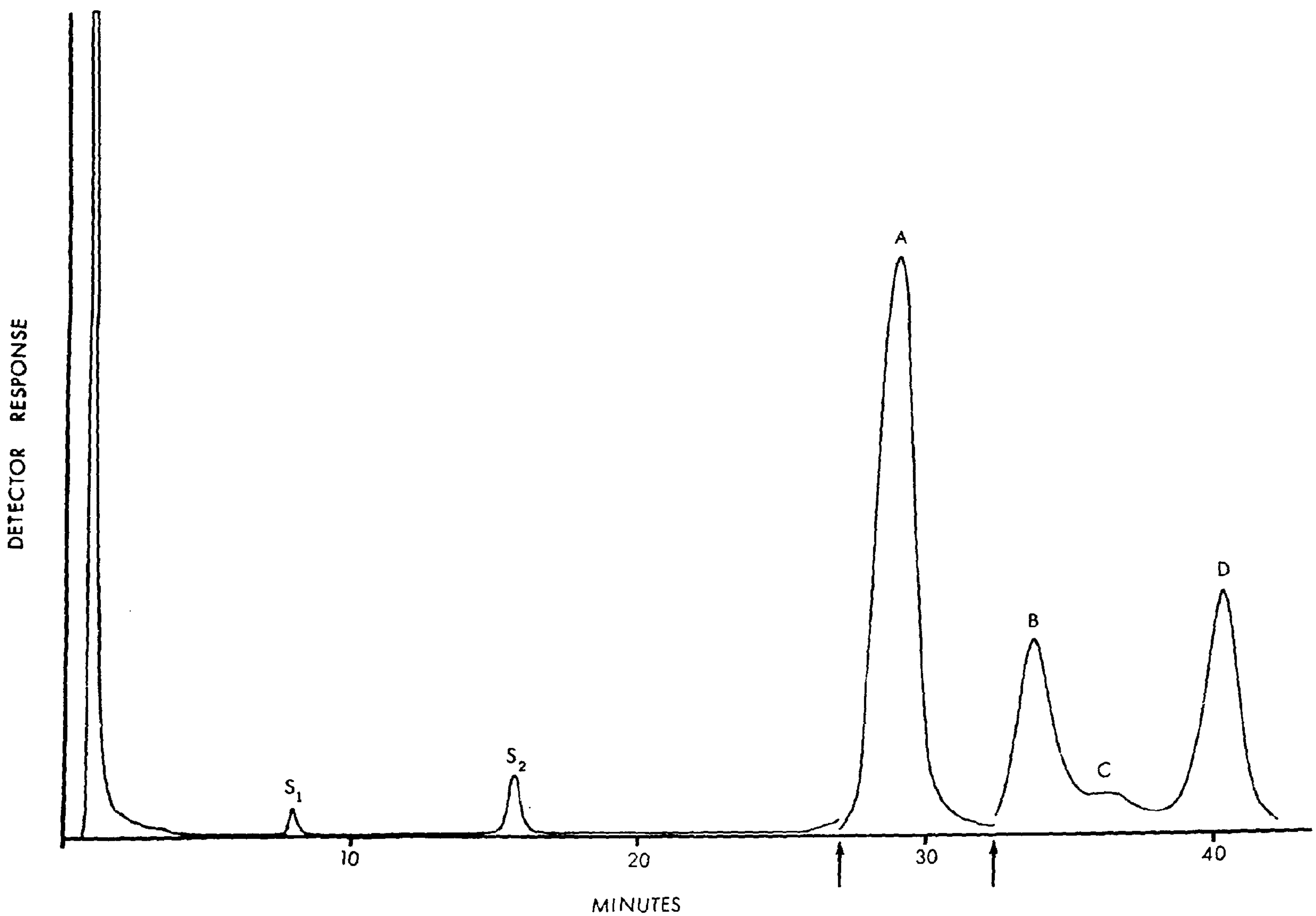
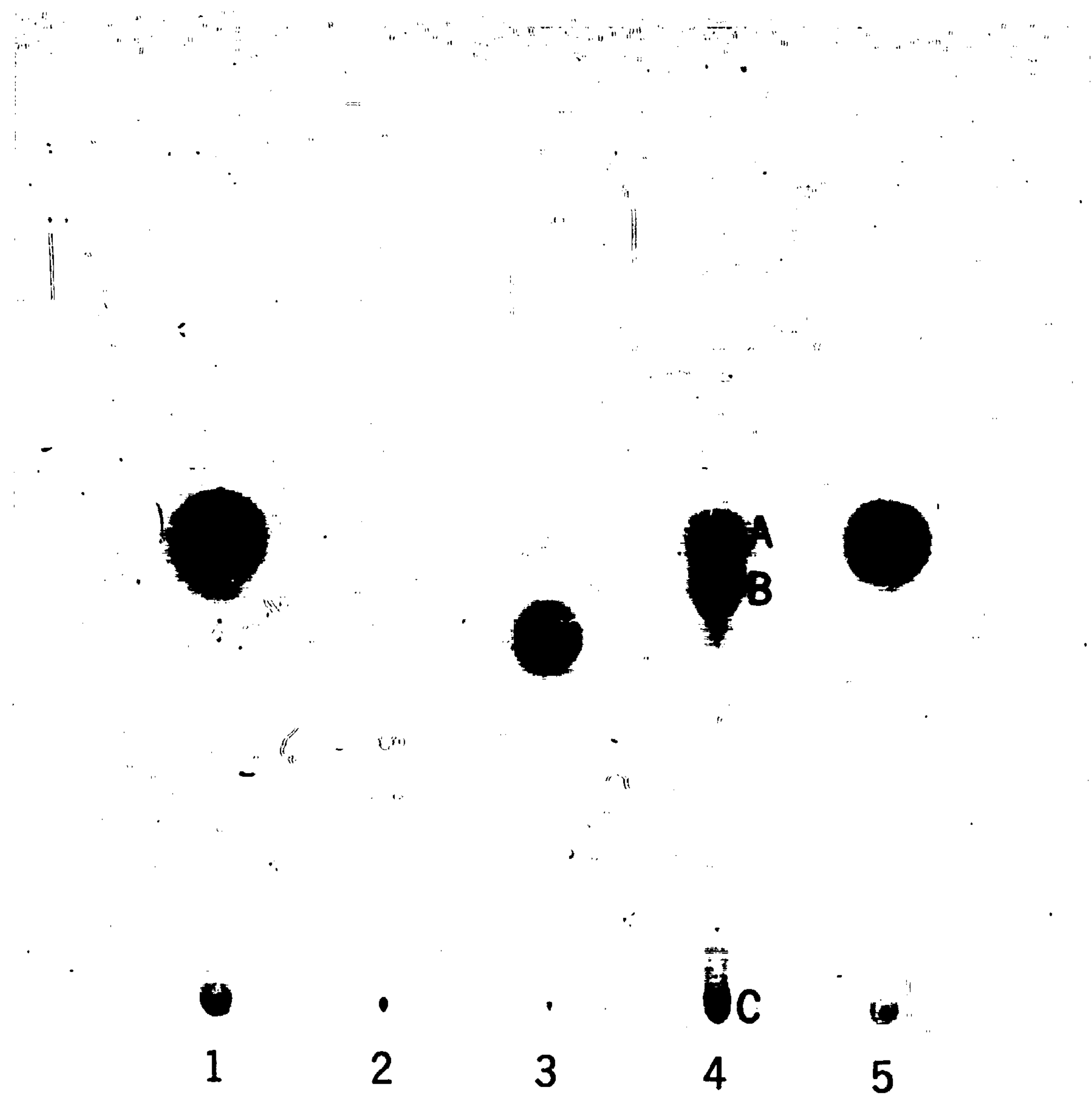

DETECTOR RESPONSE
S1
S2
A
B
C
D
10
20
30
40
MINUTES

FIGURE 2. Thin layer chromatogram on Silica Gel G eluted with hexane-ether 24:1. The spots were developed with chromic acid. Samples applied were 1) methyl oleate, 2) effluent blank collected from gas chromatographic column, 3) methyl 9,10-dibromostearate, 4) material eluted from gas chromatographic column after injection of methyl 9,10-dibromostearate, 5) methyl oleate.


A
B
C
1
2
3
4
5

used, 5 µg. of methyl 9,10-dibromostearate would have been detected. The nature of these substances was not investigated further.

Since the flash heater seemed the most likely place for decomposition to occur, a sample of methyl 9,10-dibromostearate was chromatographed on the polyester column at a series of flash heater temperatures. The peaks with carbon numbers 18.4, 18.9, 20.2 and 20.8 (see Table I) were seen at flash heater temperatures as low as 277°C. At this temperature, there was considerable peak broadening due to slow volatilization of the sample. When the flash heater temperature was lowered still further, the resolution decreased to such an extent that the chromatograms could not be evaluated.

The standard bromination method (2) for eliminating unsaturated components for gas chromatographic analysis, therefore, can give seriously misleading results if used to characterize unknown components in fatty acid mixtures. We have eliminated these difficulties by brominating the mixed methyl esters on a thin layer plate using a procedure similar to one developed by Kaufmann (4) for qualitative analysis of fatty acids by thin layer chromatography. The sample is spotted along a preparative plate of Silica Gel G and eluted with hexane-ether 19:1 containing 0.5% bromine. The plate is removed from the chamber and the

solvent is allowed to evaporate. After the positions of the components have been located by brief exposure to iodine vapor, the most rapidly migrating band is marked, scraped into a sintered glass funnel and extracted with chloroform. This extract contains saturated esters free of unsaturated esters. The procedure is simple and rapid and, if isolation of the unsaturated esters is not desired, compares favorably with methods involving the use of mercury adducts (5) or thin layer chromatography on plates impregnated with silver nitrate (6). An added advantage of the procedure is that the esters of polar acids, such as hydroxy acids, remain near the origin and can be separately analyzed. Figure 3 shows the separation achieved with a model mixture of simple esters. If bromine is omitted from the eluant, no separation of the unsaturated from the saturated esters occurs.

An example of the application of this technique is given in Table 2. A sample of pooled rat epidermal lipids was saponified with 0.5 N KOH in methanol and the fatty acids were esterified in 4% H_2SO_4 in dry methanol. The methyl esters were analyzed by gas chromatography on an ethylene glycol glutarate column. The fatty acid composition is given in terms of the percent peak areas (uncorrected). The fourth column gives the gas chromatographic analysis obtained after the sample had been treated with bromine by the procedure of Farquhar (2). The peaks with carbon numbers 16.5, 18.4 and 19.1, representing palmitoleate, oleate and linoleate respectively, were decreased but not

FIGURE 3. Separation of saturated from unsaturated fatty acid esters by thin layer chromatography on Silica Gel G using hexane-ether 19:1 with 0.5% bromine as eluant. The spots were developed with chromic acid. Samples applied were: 1) methyl palmitate, 2) methyl laurate, 3) methyl myristate, 4) methyl palmitoleate, 5) methyl palmitate, 6) mixture of 1-5, 8, 9, 11 and 12, 7) methyl palmitate, 8) methyl stearate, 9) methyl oleate, 10) methyl palmitate, 11) methyl arachidate, 12) methyl linoleate.

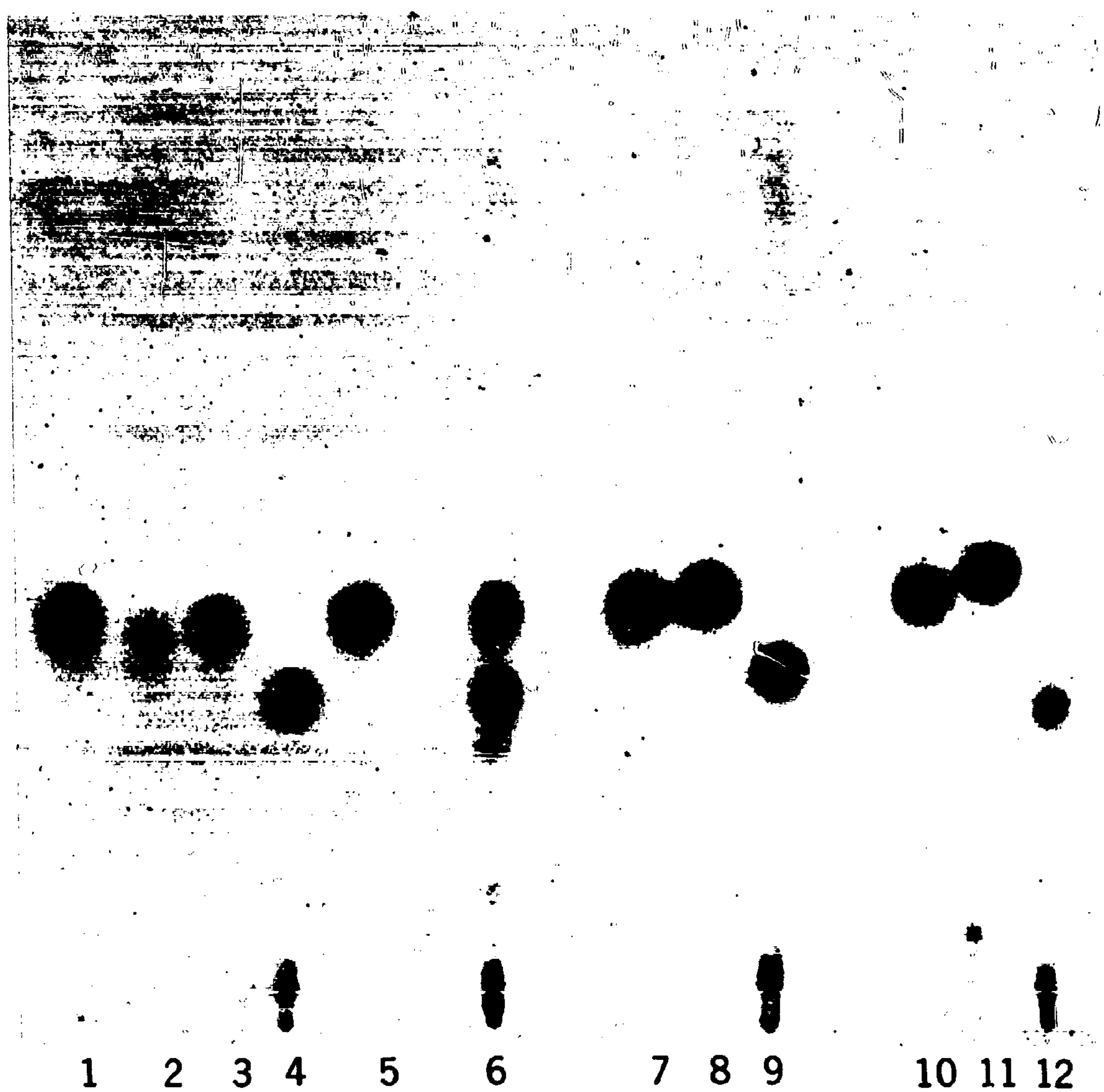

1 2 3 4 5 6 7 8 9 10 11 12

Table II

MAJOR COMPONENTS OBSERVED IN MIXTURE OF METHYL ESTERS OF FATTY ACIDS FROM RAT EPIDERMAL LIPIDS BEFORE AND AFTER BROMINATION

		Percent Peak Area		
Carbon Number[b]	Tentative Identification	Prior to Bromination	After Standard Bromination	After Bromination on Thin Layer Plate
15.0	C15:0	0.9	1.4[a]	1.0
15.6	C16:0br	1.1	1.4[a]	1.3
16.0	C16:0	19.6	33.4	56.3
16.5	C16:1	9.4	2.9[a]	-
16.8	C17:0br	-	-	1.9
17.0	C17:0	0.8	1.7[a]	1.4
17.6	-	1.7	1.2[a]	0.8
18.0	C18:0	5.0	9.3	12.7
18.4	C18:1	31.8	7.0[a]	-
19.1	C18:2	6.0	0.9[a]	0.1
19.7	-	0.8	1.4[a]	0.9
20.2	-	1.2	2.5[a]	1.8
20.5	-	3.5	0.5[a]	-
20.8	-	1.1	3.4[a]	0.9
21.0	-	1.5	0.8	-
22.1	C22:0	1.2	2.9[a]	2.4
24.0	C24:0	4.2	9.9	12.8

[a]Peak partly or entirely due to brominated adducts, as shown by gas chromatographic analysis of the brominated fraction isolated after thin layer chromatography.

[b]Methyl esters chromatographed on ethylene glycol glutarate column at 180°C.

eliminated, while the peaks with carbon numbers 20.2 and 20.8 were increased by the decomposition products of the brominated esters. The last column gives the results obtained when the saturated esters were isolated by bromination on a thin layer plate before analysis. The unsaturated esters are eliminated and saturated components, such as the branched ester with carbon number 16.8, which had previously been obscured by the unsaturated esters, have now become measurable.

We have similarly extracted the thin layer band containing the brominated esters and subjected this fraction to gas chromatographic analysis. The resulting chromatogram had a variable high base line and was not suitable for quantitative evaluation of the unsaturated esters. It was of interest, however, that peaks corresponding to a number of unsaturated esters present in the original sample were observed in this fraction, indicating that the behavior described for methyl 9,10-dibromostearate is quite general.

We have used this procedure to analyze the fatty acids of four epidermal lipid fractions (see Reference ~ble 4-I) of EFA-deficient and control rats, as well as the total epidermal lipids of vitamin B_6-deficient and control rats. These data were used to obtain the identifications previously reported.

Discussion

In our investigation of skin lipid composition, we found that the brominated derivatives of methyl esters of unsaturated fatty acids decompose during gas chromatographic analysis yielding, as major products, materials having retention times identical or close to those of the parent unsaturated esters. Landowne and Lipsky (7) have reported similar results from a brominated sample of methyl oleate. These workers suggested that the largest peak was probably methyl 9,10-dibromostearate. The retention time of this peak, however, corresponded closely to that of methyl oleate and it would be surprising if a compound of such high molecular weight and relatively low volatility would have such a low retention time.

We have collected the effluent corresponding to these peaks from the gas chromatographic column and analyzed it by thin layer chromatography. Three major components were found, one of which migrated at a rate corresponding to that of methyl oleate. These components could not be identified from the available data. However, it was shown that none of them contained significant amounts of bromine.

Any possibility of incomplete bromination was eliminated by the fact that repeated bromine treatment of the samples caused no further decrease in the peak ascribed to methyl oleate. This was observed on both Apiezon L and ethylene

glycol glutarate columns. Confirmatory evidence for this conclusion was derived from the results obtained after bromination on the thin layer plate. In the hexane-ether solvent systems we have used for thin layer chromatography, methyl oleate is not separated from the saturated methyl esters. Any unbrominated methyl oleate would therefore have remained as a contaminant of the saturated ester fraction. This fraction, however, was seen to be free of methyl oleate (Table 2). On the other hand, a peak ascribable to methyl oleate was observed when the brominated ester fraction was subjected to gas chromatographic analysis.

Our work involved analyses of mixtures containing components having long retention times. This necessitated the use of relatively high temperatures which may have contributed to the observed decomposition. Keily and Garcia (8) have shown that 1-decyl bromide and 1-dodecyl bromide also decompose when subjected to gas chromatographic analysis using conditions similar to those here reported. The decomposition of these alkyl bromides was strongly dependent on temperature and was found to be slight at a flash heater temperature of 197°C. and a column temperature of 183°C. Such low temperatures cannot be used for the analysis of very long chain fatty acid esters.

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8. STUDIES ON HUMAN SKIN: ELECTRICAL CONDUCTIVITY MEASUREMENTS

Contents

During the course of a large number of experiments with animals as subjects, changes in water permeability of skin and in its electrical conductivity showed a close correspondence (1 - 4). From our own observations and those of others, *e.g.* (5), conductivity of itself is a good means of measuring barrier integrity. The method is particularly suitable for *in vivo* studies since it is rapid and nontraumatic.

In the subsections to be presented, skin conductivity measurements were made on human volunteers. A preliminary study of conductivity at different sites showed that the areas of greatest sweat gland concentration gave the highest conductivities, and that at these sites conductivities were therefore independent to some extent of the epidermal thickness. When the outer layers of the stratum corneum were removed by stripping with cellophane tape, the conductivity response was similar to that observed by Blank (6) when he

studied the effect of tape stripping of the corneum on the water diffusion rate of excised skin. In our study of corneal stripping, recovery was more rapid than had been observed by Matoltsy and his co-workers (7) who used an *in vivo* measurement of water loss, but this difference may have been due to variation in the degree of corneal removal in the two studies.

I. Electrical Conductivity of Various Skin Sites of Normal Human Subjects: A Preliminary Study

Experimental Procedure

Male volunteers were used in this study. The skin conductivity of each subject was measured on each of 34 sites; measurements were taken using our standard procedure (1), which employs plastic-encased zinc electrodes and an electrode gel containing agar, zinc sulfate, glycerine and water. The skin site to be measured is given a single wipe on each of two proximate but nonoverlapping areas with a cotton ball wetted with 95% ethanol. This wiping serves to eliminate any continuous moisture film on the skin surface, but it also appears necessary for obtaining valid readings under dry skin conditions. The electrodes are placed on the discrete wiped areas and the conductivity measured at 1 volt (or less, if the conductivity exceeds the scale reading of 15 microamperes). Neither the distance between the electrodes nor the amount of pressure applied on the skin surface seems to influence the reading.

Although there were originally five men on the panel, the data obtained from one was rejected because of visible

sweating. In future studies, efforts will be made to control the humidity and temperature of the room in which the conductivity measurements are made.

Results

The averages for the values of the four panel members are given in Table I; the individual values are given in Table III in the Appendix to this section. In the calculation of standard deviation, N was 8 when the right and left sides (or components) of a region were measured, *e.g.*, the cheeks or the arms; N was 4 when the site was considered as a single area, as in the case of the forehead. The distinction is indicated in Table I.

Table I

AVERAGED SKIN CONDUCTIVITY VALUES OF VARIOUS ANATOMICAL SITES OF MALE SUBJECTS

Site	Conductivity µamp./volt	Site	Conductivity µamp./volt
Ear, pinna	1.2 ± 0.1[a,b]	Scrotum	10.6 ± 8.3*
" , lobe	3.5 ± 1.4	Axilla	6.7 ± 1.7
Face, forehead	5.3 ± 1.7*	Arm, inner	2.1 ± 0.9
" , cheek	4.7 ± 1.3	" , outer	1.7 ± 0.8
" , infranasal	10.7 ± 1.9	" , flexure	2.2 ± 1.3
" , lip (lower)	6.6 ± 4.0	Forearm, volar	2.2 ± 1.4
Neck, nape	3.1 ± 2.9*	" , outer	1.7 ± 0.9
" , side	3.0 ± 2.2	Hand, back	2.7 ± 1.4
Throat	2.3 ± 2.0*	" , palm	5.6 ± 2.6
Chest, supra-alveolar	1.9 ± 1.5	Thigh, anterior	2.4 ± 0.9
Abdomen, supra-navel	3.2 ± 1.8	" , posterior	1.9 ± 0.4
" , sub-navel	3.9 ± 3.0	Knee	3.5 ± 0.5
Back, scapular	3.0 ± 1.4	Poples	2.1 ± 0.6
" , thoracic	3.4 ± 1.5	Calf	1.8 ± 0.2
" , lumbar	3.2 ± 1.0	Shin	2.7 ± 0.9
Buttocks	2.7 ± 0.8	Foot, metatarsus	4.7 ± 1.0
Groin	3.8 ± 1.9	" , sole	5.8 ± 1.1

[a]Means and standard deviations. N is 8 except where indicated by an asterisk, when N is 4 (see text for discussion).

[b]In the room in which measurements were made, temperature was about 80°F., relative humidity about 44%.

Discussion

On the basis of the magnitude of electrical conductivity measurements, it is convenient to assign each skin site to one of three general groups:

Group I includes those areas which exhibited relatively high values, namely the infranasal portion of the face, and the scrotum. The large standard deviation of the latter measurement should be noted.

Group II includes the areas whose conductivity values were somewhat lower than those observed in Group I. The forehead, cheek, lip, axilla, palm of hand, and metatarsus and sole of the foot fall into this category.

Group III includes areas showing values which lie within the range that we have observed in previous studies on normal animals. The sites not mentioned as belonging to Groups I and II are assigned to this category.

The thickness of the epidermis of the various areas of the body that we have measured varies greatly (8), and appears to be unrelated to the results of the conductivity measurements. From comparison of our results with the data of other investigators (9, 10), there seems to be a consistent relation between the electrical conductivity of a given skin site and the density of sweat glands (Appendix, Table IV), but no relation to the relative intensity of sweating (Appendix, Table V) at that site.

It may be seen by reference to Table III (Appendix) that for each site there was good agreement between the bilateral conductivity values of each individual, although for many sites there was considerable variation between individuals.

The present study provides no estimate of day-to-day variation for a given individual. The study will be expanded to include a greater number of subjects, a greater frequency of measurements on each individual, and more stringent definition of the variables encountered in such a study.

II. Effect of Cellophane Tape Stripping of Corneum on the Electrical Conductivity of Human Skin

Experimental Procedure

Six adult males were subjected to successive strippings of the corneum layers of the epidermis using Scotch-brand cellulose tape. A strip of one inch tape about six inches long was used for each stripping. The tape was applied to a demarcated area on the volar surface of the forearm, massaged by five strokes with the heel of the hand, using moderately heavy pressure, and was removed in one swift motion. The direction in which the tape was removed was alternated for each stripping; conductivity was measured after every other stripping. End points were reached when a "high" reading (15 - 25 μamps./volt) was reached, at which point localized shiny areas were evident. Several

recovery measurements were also taken.

Results and Discussion

The conductivity data for each of the subjects are given in Table II and are shown graphically in Figure 1.

Table II

EFFECT OF SCOTCH TAPE STRIPPING OF CORNEUM ON HUMAN SKIN CONDUCTIVITY

No. of Strippings	Electrical Conductivity (μamp. volt^{-1})					
	E.S.*	J.S.	H.F.	P.W.	A.M.	L.C.
			Injury Phase			
0	2.4	1.2	1.4	3.8	2.8	1.6
2	4.6	2.0	3.2	4.2	1.2	2.8
4	5.0	2.8	3.4	4.4	3.4	2.8
6	5.2	3.8	3.2	4.4	3.4	3.0
8	5.2	2.8	3.8	4.6	5.2	4.0
10	5.6	3.0	4.0	5.4	4.0	3.6
12	6.2	2.2	3.4	6.0	3.2	4.2
14	7.6	2.8	5.2	6.0	3.8	5.2
16	13.2	4.4	4.0	5.8	5.6	4.8
18	23.0	3.8	4.8	5.4	5.4	4.6
20		5.8	6.8	8.0	5.2	4.2
22		17.2	6.0	8.4	6.6	5.0
24			6.6	7.2	7.6	6.0
26			11.0	7.8	4.8	3.6
28			16.0	14.6	9.4	3.2
30				19.6	20.8	4.2
Time Post Stripping			Recovery Phase			
3 hours	24.0	7.8	7.6	16.8	13.2	-
22 hours	5.4	3.4	2.0	3.6	3.8	-
100 hours	1.6	1.4	2.2	3.4	1.2	-

*Designation of subjects.

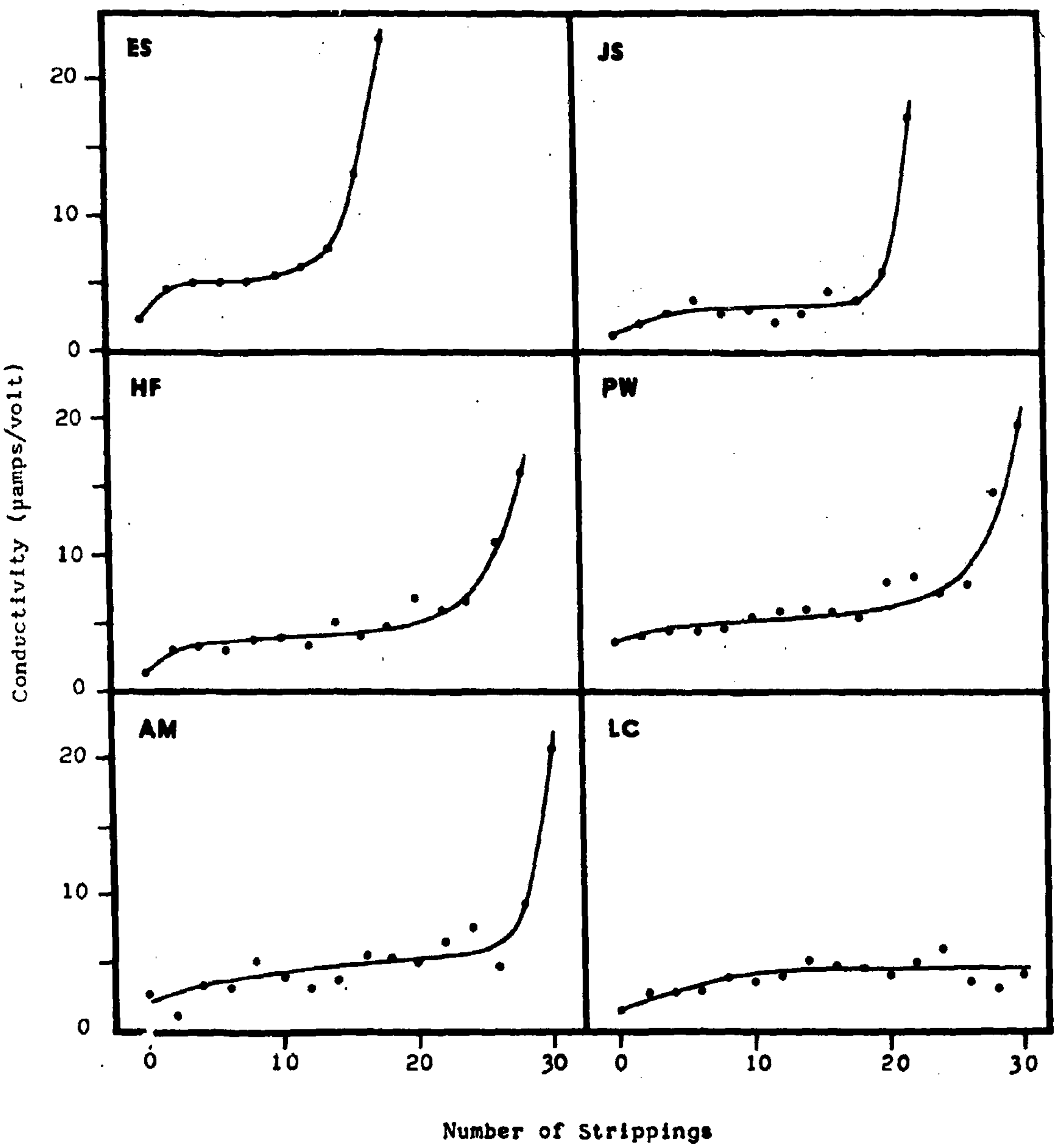

FIGURE 1. Electrical Conductivity Changes Occurring During Tape Stripping of the Outer Epidermal Layers of Human Volunteers.

The sharp increase in conductivity noted (Table II) in the skin of certain subjects following the first stripping (particularly E.S., H.F., L.C.) is interpreted to be the result of increased blood flow to the area in response to the treatment. After the intial increase, a gradual upward trend is seen in the conductivity values, followed by a sharp increase. The stepwise line in the table is used to show the point at which, it is judged, true breaching of the barrier has begun to occur. One subject, L.C., did not reach an end point within 30 strippings, so the treatments were discontinued.

Recovery from the treatments was fairly rapid in all subjects. Two of the subjects (J.S., H.F.) showed a decrease of about 50% in skin conductivity within three hours, and one (H.F.) gave a near-normal reading after only 22 hours. The damage to the skin of this subject (H.F.) by the stripping may have been somewhat less than in the case of the other subjects.

The rate of recovery observed in this study is considerably greater than was observed by Matoltsy _et_ _al_. (7) following adhesive tape stripping of skin. In two of his three subjects, there was virtually no decrease in water permeability on the day after the stripping with only slightly better results in the third individual. In contrast, we found a significant improvement in all subjects 22 hours

after the treatment. The extent of injury may have been greater in Matoltsy's study - in our study the stripping was not continued to the point at which the entire skin surface had a shiny appearance.

The curves plotted in Figure 1 are very similar in type to the curve published by Blank (6) for water diffusion through human skin from which the corneum was stripped after excision. Blank found a sharper rise from the base level value than we have observed, however. It is not unlikely that the difference can be explained on the basis of an *in vivo* skin reaction to trauma, a problem not met in Blank's study on excised skin.

Differences that cannot be easily accounted for exist between our observations and those of Lawler *et al*. (12) and of Loveday (13). The former group measured the resistance and capacitance of skin of the volar surface of the forearm of normal subjects, and included in their study measurements of skin resistance, using alternating current, during the process of cellulose tape stripping of the corneum. Under conditions we believe comparable to ours, the skin resistance was found to decrease steeply during the first 13 strippings, and then to level off gradually. In Loveday's study of salicylic acid penetration through skin of swine ears, Scotch tape stripping resulted in a relation somewhat similar to that reported by Lawler *et al*. - that each stripping was about as effective as the next in reducing the barrier properties of skin.

APPENDIX

Table III

INDIVIDUAL SKIN CONDUCTIVITY VALUES OF VARIOUS ANATOMICAL SITES OF MALE SUBJECTS

Site	Electrical Conductivity (μamp. volt^{-1})							
	Subject I		Subject II		Subject III		Subject IV	
	Left	Right	Left	Right	Left	Right	Left	Right
Ear, pinna	1.1	1.2	1.1	1.1	1.2	1.1	1.1	1.4
" , lobe	6.2	4.2	1.9	3.4	1.9	3.2	3.8	3.7
Face, forehead	4.8		4.9		3.8		7.8	
" , cheek	3.7	5.9	2.9	3.0	5.5	5.1	5.4	5.8
" , infranasal	12.4		9.5		8.6		12.2	
" , lip (lower)	4.7		5.3		12.5		3.8	
Neck, nape	1.0		3.0		1.1		7.2	
" , side	0.8	1.2	2.9	4.1	1.4	1.0	5.8	6.4
Throat	1.1		2.0		0.8		5.2	
Chest, supra-alveolar	0.7	0.8	2.0	2.0	0.7	0.7	4.0	4.3
Abdomen, supra-navel	3.4	2.9	2.3	2.7	1.4	1.3	6.0	5.9
" , sub-navel	2.0	3.0	2.8	2.5	2.4	1.6	8.8	8.7
Back, scapular	2.0	2.6	1.7	2.8	2.5	2.1	5.3	5.3
" , thoracic	2.7	2.9	3.0	3.5	2.1	1.6	4.9	6.1
" , lumbar	3.0	3.2	2.1	2.0	2.7	2.8	4.5	4.9
Buttocks	2.8	3.0	2.0	2.2	2.2	2.1	3.4	4.1
Groin	2.5	3.1	2.6	2.7	2.4	3.1	6.7	6.9
Scrotum	14.9		2.4		4.9		20.0	
Axilla	6.5	8.4	4.7	7.4	5.0	5.2	7.1	9.2
Arm, inner	3.2	2.9	2.1	1.9	0.6	1.1	2.4	2.4
" , outer	2.3	1.0	1.8	1.5	0.9	0.7	2.6	2.6
" , flexure	3.3	3.8	1.4	1.4	0.8	0.7	3.4	2.9
Forearm, volar	4.4	4.2	1.5	1.6	0.7	0.7	2.5	1.7
" , outer	3.0	2.8	1.4	1.2	0.6	0.7	1.5	2.0
Hand, back	4.2	4.1	1.8	2.8	0.9	1.0	3.9	3.1
" , palm	4.8	6.2	3.8	3.3	3.9	4.1	10.2	8.8
Thigh, anterior	2.1	1.9	2.3	2.2	1.9	1.5	3.8	3.7
" , posterior	1.4	1.8	1.8	1.9	1.5	1.7	2.5	2.6
Knee	3.7	4.1	3.8	3.0	2.9	2.9	4.0	3.7
Poples	1.5	2.0	1.9	1.7	1.6	1.7	3.2	2.9
Calf	1.9	1.7	1.8	1.6	1.6	1.6	2.2	2.1
Shin	2.1	1.8	2.8	2.9	2.2	2.3	3.2	4.5
Foot, metatarsus	5.0	5.0	3.8	3.6	4.0	3.7	5.6	6.5
" , sole	5.2	6.0	5.4	5.5	4.6	4.8	7.6	7.0

Table IV

DISTRIBUTION OF ECCRINE GLANDS IN MAN (9)

(Figures Indicate Number of Sweat Glands Per Square Inch of Surface Area)

Palms	2,736
Soles	2,685
Dorsa of hands	1,490
Forehead	1,258
Chest and abdomen	1,136
Forearm	
Flexor aspect	1,123
Extensor aspect	1,093
Dorsa of feet	924
Thigh and leg, medial aspect	576
Thigh, lateral aspect	554
Cheek	548
Nape of neck	417
Back and buttocks	417

Table V

RELATIVE INTENSITY* OF SWEATING OVER VARIOUS REGIONS OF BODY SURFACE (10)

	Subject P.	Subject McK.	Subject W.
Head	1.4	1.4	1.0
Trunk	1.4	1.5	1.3
Thighs	0.8	0.5	0.8
Legs	0.9	0.7	1.2
Feet	0.7	0.4	0.2
Arms and forearms	0.6	0.8	0.7
Hands	0.2	0.2	0.3

*Relative intensity = Proportion of total sweat contributed by region ÷ proportion of total surface represented by region.

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9. DIFFUSION OF WATER THROUGH SKIN MODEL MEMBRANES

Contents

It has been demonstrated that the moisture barrier of the epidermis is destroyed by treatment whose primary effect is the thorough removal of lipids from the tissue and that the barrier properties can be restored *in vitro* by replacement of the extracted fatty matter. The literature on water permeability of organic substances such as lipids deals almost exclusively with monolayers spread on water. The work described in this section was done to provide information on the effect of various physical and chemical factors on the bulk permeability of substances related to those likely to be found in the epidermis.

The theory of the measurements is discussed at some length to provide a basis for correction for the finite rate of evaporation of water and for diffusion resistances other than that of the membrane.

Theoretical

The molecules of an ideal gas at pressure p,* collide with the walls of the container at the rate $p/(2\pi mkT)^{\frac{1}{2}}$ molecules/ $cm.^2$/sec., where m is the mass of a molecule. In a system consisting of a pure liquid in equilibrium with its vapor at pressure p_o, the necessarily equal rates of condensation and evaporation are given by:

1. $F_o = p_o/(2\pi mkT)^{\frac{1}{2}}$ molecules/$cm.^2$/sec., on the assumption that every vapor molecule is captured by the liquid on contact. This is the theoretical maximum rate of evaporation of the liquid into a perfect vacuum (condensation rate zero). For water at 25°C., F_o = 0.34 gm./$cm.^2$/sec. or 1.2×10^6 mg./$cm.^2$/hr. The experimentally determined rate of evaporation of water, F, is 0.013 gm./$cm.^2$/sec. or 4.8×10^4 mg./ $cm.^2$/hr. The ratio, $\alpha = F/F_o = 0.039$, is called the condensation coefficient since it is inferred that only this fraction of vapor molecules which impinge on the liquid surface condense, the remainder being reflected (1).

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*See Table I for list of symbols.

If the pressure of the vapor is maintained at some value, p, the rate of condensation from the vapor phase will be $\alpha p/(2\pi mkT)^{\frac{1}{2}}$ and the net rate of evaporation or the rate of loss of mass of the liquid phase under these conditions will be

2. $F = \alpha(p_0 - p)/(2\pi mkT)^{\frac{1}{2}}$ molecules/cm.^{2}/sec.

Substituting $p = nkT/v = ckT/m$ where c is the concentration of vapor in grams/cm.^{3}, and multiplying by m gives

3. $F = \alpha(kT/2\pi m)^{\frac{1}{2}}(c_0 - c)$ gm./cm.^{2}/sec.

4. $F = \alpha Q(c_0 - c)$ gm./cm.^{2}/sec.

where $Q = (kT/2\pi m)^{\frac{1}{2}}$ cm./sec. and c_0 is the saturation concentration of vapor.

If instead of evaporation into a vacuum, we consider one-dimensional, steady state diffusion through an air column from the liquid surface to a desiccant, we find that the diffusion process is rate controlling for any experimentally feasible air gap. Fick's first law of diffusion

5. $J = -D\,\partial c/\partial x$

can be applied in the form

6. $J = Dc/L$ where J is the flux of water vapor, D is the diffusion coefficient, c is the concentration of vapor at the liquid surface and L is the path length from the water surface to the surface of the desiccant which is assumed to maintain a negligibly small water vapor concentration.

The diffusion coefficient of water vapor in air at pressure P atmospheres, temperature T°K is given (2) as

7. $D = \frac{0.220}{P} (T/273)^{1.75}$ cm.^{2}/sec.

For one atmosphere pressure at 25°C., D = 0.256 cm.^{2}/sec.

Under steady state conditions the rate of evaporation must equal the diffusion flux, i.e., F = J. From equation 4 and 6

8. $-Dc/L = \alpha Q(c_o - c)$ whence

9. $c = \frac{\alpha QL}{D + \alpha QL} c_o$

10. $c = \frac{L}{L+b} c_o$ gm./cm.^{3} where $b = D/\alpha Q = 4.44 \times 10^{-4}$ cm. at 25°C., one atmosphere.

b is the air diffusion path length equivalent in resistance to the liquid-vapor interface. Evidently,

if L is of the order of one centimeter, this increment is negligible and the process is diffusion controlled.

It is interesting to note in passing that if we calculate b from the coefficient of self-diffusion of water, we obtain a value of only 4×10^{-8} cm. or roughly twice the diameter of a water molecule.

We can now formally represent the liquid surface as an air column of length b whose high pressure face is maintained at saturation.

If there are other diffusion elements present whose surfaces are planes parallel to the liquid surface (maintaining the simple, one-dimensional geometry of the diffusion field), the flux through each must be the same in the steady state and the sum of the concentration increments across all the elements must be c_o, the saturation concentration.

If we make two simplifying assumptions regarding these elements, we can assign a diffusion resistance to each and treat the composite barrier as an analog of an electrical circuit of resistances in series. These assumptions are, first, that the solubility of water in the element is proportional to the partial pressure (or concentration) of water vapor, and second, that the diffusion coefficient is independent of concentration.

Under these assumptions, we can apply Fick's law again in the form.

11. $J = -D\,\Delta S/\Delta X = Dk\,\Delta c/\Delta X$ where $kc = S$ is the solubility of water in equilibrium with vapor of concentration c, k is the appropriate Henry's law constant and ΔX is the thickness of the element. Then

12. $c = -J\,\Delta X/kD$, and for the entire system

13. $\Sigma\,(\Delta c)_i = c_o = J(1/\alpha Q + \Sigma\,(\Delta X)_i/k_iD_i)$ where $k = 1$ for an air gap. The analogy to Ohm's law as applied to a circuit of resistances in series,

14. $\Sigma\,E_i = E_{app} = I\,\Sigma\,R_i$

is evident; here E_{app}, the total applied voltage, corresponds to c_o, the total concentration drop across the composite, the current, I, corresponds to the flux, J, and the terms $1/\alpha Q$ and $(\Delta X)_i/k_iD_i$ correspond to the individual resistances, R_i.

It is evident again that the evaporation resistance, $1/\alpha Q = 1.73 \times 10^{-3}$ sec./cm. is negligible compared to the air diffusion resistance, $(\Delta X)air/D_{air} = 3.91\ (\Delta X)_{air}$ sec./cm. for all but the most minute air gaps. Terms

representing solid or viscous liquid membranes in which the diffusion coefficients are of the order of 10^{-4} to 10^{-9} and in which the solubility of water is low, will, in turn, overshadow the air resistance and become rate controlling. The monomolecular films currently being studied for reservoir evaporation suppression are an exception because of their extreme thinness. For example, a monofilm of octadecanol at a surface pressure of 40 dynes/cm. has a thickness of about 2×10^{-7} cm. and a resistance of about 4 sec./cm. at 25°C. (3). This resistance exceedsthat of a pure water surface by a factor of 2.3×10^{3} and would reduce the rate of evaporation into a vacuum by the same factor, but in series with an air gap of one centimeter (which has the same resistance as the monofilm) the evaporation rate would be reduced by only 50 percent.

The assumptions leading to equations 11 to 13 and to the assignment of a resistance value to each element are not generally valid. The solubility of water is usually not a linear function of vapor concentration and the diffusion coefficient, in most cases, depends strongly on the local concentration. When this is the case, the diffusion resistance, $\Delta X/kD$, of a particular element will depend on the position of the element in the series as well as on the total vapor pressure differential applied to the composite barrier. The flux through such a barrier will not generally be proportional to the applied pressure differential and may change when the direction of the pressure drop is reversed (4).

In particular, the permeability of keratin is strongly dependent on its degree of hydration; the permeability remains quite low up to a relative humidity of 70% and then increases sharply. From King's data (5), a 50 micron thick membrane of horn keratin transmitted water vapor at about 0.2 mg./cm.^{2}/hr. when the relative humidity was 70% on one side, zero on the other; when the relative humidity was increased to 90% on the wet side, the flux increased to about 4 mg./cm.^{2}/hr. and continued to increase approaching a value of about 8 mg./cm.^{2}/hr. at saturation.

Evidently, a layer of keratin might introduce an appreciable water diffusion resistance at the dry or low-pressure end of a composite barrier but might have negligible resistance near saturation at the high pressure face of the same composite.

It is possible that the diffusion barrier in skin is heterogeneous with respect to phase. Diffusion through crystalline solids is so much slower than through liquids that it may usually be assumed that all transport in a mixture takes place through the latter, if it is the continuous phase. When this is the case, the permeability is determined by the shape and volume fraction of the solid particles (6) but does not depend on their absolute size. Since the direction of the concentration gradient and flux are partially randomized in a heterogeneous medium, the diffusion

process is no longer strictly one dimensional but it is usually treated as such by taking the volume fraction of liquid or amorphous material as the cross-sectional area available for linear transport and introducing an empirical tortuosity factor to take account of the increase in the effective diffusion path length relative to the measured thickness of the membrane. For a suspension of nearly spherical particles, the tortuosity factor is of the order of unity (6) but may be as great as 30 for highly anisometric particles.

In an anisotropic solid, the diffusion coefficient may vary strongly with direction with respect to the crystal axes (and in this case, the flux and concentration gradient vectors are not necessarily collinear). Although no data have been found in the literature on diffusion in liquid crystals, it is quite reasonable to suppose that the one or two dimensional order prevailing in these phases would make them anisotropic with respect to diffusion with low diffusion coefficients in the direction of the long axes of the molecules. Indirect evidence for this is the fact that the evaporation resistances of liquid condensed monolayers is independent of surface pressure over a wide range; this is interpreted by Archer and La Mer (7) as indicating that the chain-chain interaction energy is independent of the angle of tilt with respect to the interface. For a number of chain lengths, evaporation resistance of the

liquid condensed phase was found to be roughly half that of the solid surface phase. Evidently, perfect order in the chain packing is not essential for high resistance. It has been reported (8) that a monofilm of wax in the cuticle of certain insects offers more resistance to evaporation than a considerably greater thickness of the same wax in a more disordered state. Cuticle permeability was found to decrease sharply and irreversibly at a certain temperature presumably due to an irreversible disordering of the protective layer. Sebba and Briscoe (9) found that it was possible to obtain greater evaporation resistance with a close packed monofilm than with a film of lubricating oil of the order of 100 molecules thick.

Condensed surface monofilm states may be considered two-dimensional smectic states (10) since they resemble this state of matter more closely than the isotropic liquid state. A properly oriented thin film of lipid might then provide evaporation resistance comparable to that of the same mass of crystalline solid while retaining the interesting and possibly biologically advantageous mechanical properties of the homoeotropic texture of the smectic state (see Gray (10) pp. 18 to 23). In this texture, the layers of oriented molecules slip over each other extremely easily but offer much greater resistance to shearing forces normal to the planes.

Apparatus and Method

The water diffusion chamber described in detail in reference No. 11 was used for all permeation measurements. It is shown schematically in Figure 1. It consists of a cup, A, with holes to receive the prongs of the ring, B, and a locking nut, C. The membrane to be tested is pierced by the six prongs of B and held in place between parts A and B as shown. The horizontal cross-sectional area of the liquid and vapor spaces is 3.0 $cm.^2$ throughout.

About 2 ml. distilled water were placed in the cup. The cup was stuffed with tissue paper so that the water would not slosh about and damage the membrane. This also reduced the discoloration and corrosion of the aluminum foil leak-test membranes (see below) probably by preventing electrolytic contact with the aluminum alloy of the cup.

The apparatus was assembled by holding the cup in a vise and tightening the locking nut with pliers until greater torque caused visible flexing of the membrane indicating a positive pinching action. If less torque was applied, gross leaking occurred along a route under the membrane, through the prong perforations and over the membrane to the exterior at the inner edge of the pronged ring, B. Since this route is blocked by a seal of less than a tenth of an inch at each of the six prongs, considerable pressure is required to prevent leaks with tough membranes such as aluminum foil or Millipore filters.

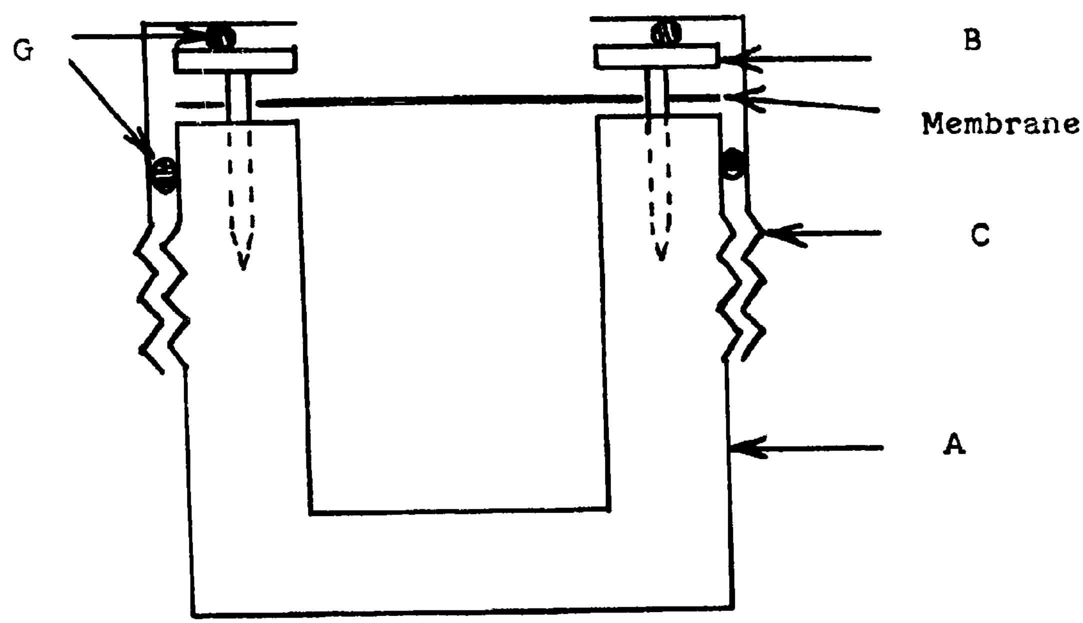

FIGURE 1. Schematic Drawing of Water Diffusion Chamber.

A - Cup

B - Ring

C - Locking Nut

G - Rubber Grommets

Aluminum foil was used as a membrane to test for leaks. Gross rates of loss in some cases were traced to pinhole corrosion which usually occurred near a prong in the region of greatest stress. Pinnoles amounting to an apparently negligible fraction of the foil area caused leakage at rates as great as one tenth that observed with an open cup. As noted above, pinhole corrosion and even superficial discoloration were eliminated by stuffing the cup with tissue.

Even with tightly sealed, intact foil membranes, leaks corresponding to permeation rates of about 0.01 mg./cm.^{2}/hr. were frequently encountered. Most of this residue was traced to dehydration of the two rubber grommets (G in Figure 1). Eight of these grommets were weighed without prior treatment and stored over desiccant. The average weight loss for the first two days was equivalent to a permeation rate of 0.013 mg./cm.^{2}/hr. (assigning 3 cm.^{2} to each set of two rings) and remained at an equivalent rate of 0.001 mg./cm.^{2}/hr. for the next 20 days. Since the most common leak route (through the prong perforations) was not blocked by the grommets and since the dry membranes used in these studies would not be expected to lose appreciable amounts of water at the outer edge of the ring, B (as might be the case with wet skin or the like), the grommets were not used after this observation. Chambers with aluminum foil test membranes without rubber grommets

lost weight at a rate corresponding to only a few ten thousandths of a milligram per square centimeter per hour for more than a week. This was deemed satisfactory.

Rates of free evaporation and evaporation through monofilms were measured directly over much shorter periods of time by placing the cup (part C of Figure 1) on the pan of an automatic projection balance. A desiccant container was placed in the weighing chamber at about the same distance from the cup as in the desiccators used in membrane permeation measurements.

In earlier work in this laboratory (12), it was found that hen's egg shell membrane and dialysis tubing were unsatisfactory support matrices for lipid membranes because of the tendency of the oil phase to be displaced in the form of nonwetting beads at high humidity. Satisfactory membranes were obtained by dipping Millipore filters in liquid oils. The weight of oil retained after wiping away the excess was quite reproducible (within 1% of the mean for a given oil). No tendency to shed the imbibed oil was noted when the membranes were in contact with liquid water. Filter pore size variation over a wide range had no significant effect on water transmission.

In the present study, all membranes were prepared from Millipore, Type VC filters, pore size 100 $\pm$ 8 millimicrons,

pore volume 74%, thickness 130 ± 10 microns. The dry weight of these filters is about 5.0 mg. per cm.^{2}; they imbibe about 5.7 mg. of oil per cm.^{2}. Occasional voids were found after dipping; these were easily detected because of their opacity and were readily filled on repeat dipping. Solids and liquid-solid mixtures were applied as homogenous melts. No attempts were made to control the rate of cooling.

Results and Discussion

In all the membrane permeability measurements performed as described in the experimental section, the membrane resistance is in series with the evaporation resistance of the water surface, the air diffusion resistance within the cup and the air diffusion resistance from the outside of the membrane to the desiccant. In the theoretical section, it was shown that the evaporation resistance is negligible but that under some circumstances the air diffusion resistance may be appreciable. It was not feasible to calculate this resistance for the desiccant chamber used since the geometry is complex, not a simple, one dimensional arrangement. To determine the order of magnitude of this contribution, rates of evaporation were measured from an open cup (part C of Figure 1) on the pan of an automatic projection balance with approximately the same geometry of diffusion path as that in the desiccator chamber.

The results of various evaporation experiments are given in Table II. Experiments 1 and 2 show that the loss rate, 36.6 $\pm$ 1 mg./cm.^{2}/hr., in air is reduced by a factor of 1300 from that expected for evaporation into a vacuum (4.8 x 10^4 mg./cm.^{2}/hr.) due to the diffusion resistance of the air. In the fourth column of Table II, the evaporation rate measurements are given in terms of the equivalent one dimensional air diffusion column length in centimeters, given by Fick's law, $L_e = D(\text{air})c_o/J = 21.2/J$ where J is the evaporation rate in mg./cm.^{2}/hr., D is the diffusion coefficient of water in air at one atmosphere, 25°C. (922 cm.^{2}/hr.) and c_o is the saturation concentration of water vapor at 25°C. (0.0230 mg./cc.).

Experiments 1 and 2 show that this parameter is about 0.6 cm. for the balance case with desiccant. This is much less than would be expected from measurement; the cup to desiccant distance is ten times as great as the equivalent value. The difference is probably due in part to the nonlinear geometry but the major contribu- is thought to be due to gentle convection currents. Even with ideal temperature control, convection would be expected because of the lower density of the moist air rising from the water surface.

In experiment 3, with the water level 1 cm. below the lip of the cup, the total equivalent path length is greater by the same amount indicating that there is little

convection within the cup.

In experiments 4, 5 and 6, the cup was covered with a Millipore filter, Type VC, held in placed by the pronged ring. Again, the total equivalent path length appears to be the sum of an exterior path, 0.9 to 1.1 cm. in this case, and the path within the cup. The increase in the exterior path over that obtained in experiment 3 is probably due largely to the extension of the nonconvective path by the ring which is 0.3 cm. thick. (The manufacturer's data on flow of air _vs._ pressure indicate that the resistance of the dry membrane would be negligible.)

The distance from the liquid surface to the lip was measured more carefully in experiments 7 and 8 from the net weight of water and the known cross-sectional area of the cup which was in this case waxed lightly on the inside to prevent the meniscus from creeping up the side. These measurements yield a figure of 1.2 cm. for the equivalent external path length, again including the effect of the pronged ring.

Experiments 9 through 12 illustrate the effect of a monofilm of hexadecanol on water evaporation rate. The fatty alcohol was spread by touching a molten drop to the water surface. Simply sprinkling the surface with small crystals had no noticeable effect on evaporation rate and it was subsequently demonstrated with a talcum

dusted surface that there was no spontaneous spreading under these conditions. The path increments 0.26 and 0.30 cm. were converted to resistances of 1.02 and 1.17 sec./cm. using the relation $R = \Delta L/D(\text{air})$ where $D(\text{air}) = 0.256$ cm.^{2}/sec. and ΔL is the increment in L_e due to the monofilm. These figures are in good agreement with the data of Barnes and La Mer (3).

In summary, the data of Table II show that the external air diffusion path makes an appreciable contribution to the total evaporation resistance (4.65 sec./cm. with the pronged ring in place, average of experiments #7 and #8) which must be taken into account in the measurement of small resistances such as that of a monofilm.

The results of permeability measurements on Millipore filters impregnated with various liquid oils are presented in Table III which gives some of the experimental details showing reproducibility, and Table IV which gives the calculations based on the mean value for each oil. The flux values given for the lightest mineral oil (0.33 poise) and the silicone oils are averages over six days; the others are averages over 13 days. The day to day variations were somewhat greater than the variations from specimen to specimen, probably due to changes in room temperature as indicated by the fact that variation in ratios of weight losses were smaller.

The total resistances due to both the membrane and the air path (fourth column of Table IV) were calculated from the relation $R_t = L_e/D = c_o/J = 82.8/J$ sec./cm. where J is in mg./cm.^{2}/hr. The membrane resistances (fifth column) were estimated as $R_m = (R_t - 5.1)$ sec./cm. allowing 4.6 sec./cm. for the external air path and 0.5 sec./cm. for that within the cup. The latter was maintained at 0.1 cm. through the wick action of the tissue with which the cup was stuffed (see Experimental Part). The air diffusion resistance varies from about 0.6% to 10% of the whole.

If the solubility of water in the various oils were known, an average diffusion coefficient of water could be calculated from the resistance and thickness of each membrane using the relation $R = (\Delta X)/kD$. In the absence of this information, we can only say that for a given chemical type of oil, the Henry's law constant, k, is probably independent of viscosity to a good approximation and that the diffusion coefficient is inversely proportional to the resistance. If the Stokes-Einstein relation were obeyed, $D = kT/6\pi vr$, where v is the viscosity of the medium and r is the radius of the diffusing particle, we would expect the ratio v/R given in Table IV, to be a constant. This is not the case. It is evident that resistance in the mineral oil series increases less rapidly than in direct proportion to the viscosity.

The remarkable absence of dependence on viscosity in the silicone oil series is in accord with the data of Barrie and Platt (13) on the water permeability of polydimethylsiloxane (which may be taken as the infinite-viscosity member of the series); from their data, a flux of 1.9 mg./cm.^{2}/hr. was estimated for diffusion of water through the polymer under the conditions of temperature and film thickness which gave a flux of 1.5 mg./cm.^{2}/hr. for the silicone oils.

The effect of a solid component (beeswax) in the oil phase (cottonseed oil) is illustrated by the data in Table V. The membrane resistance values were calculated as explained in connection with the data of Table IV. The fourth column of Table V shows the fluxes to be expected if the permeability were proportional to the volume fraction of oil, V, assuming no diffusion through the crystals. The ratio of this quantity to the observed flux is the apparent tortuosity factor given in the last column.

The high value of the tortuosity factor at high wax content is a reflection of the expected anisometry of the wax crystals. Because of the shape of the membrane and the method of preparation, there may also be an orientation factor. Examination of the completed membranes between cross polaroids revealed the presence of many crystals whose length was greater than the thickness of the Millipore Filter. Obviously, such crystals are preferentially

oriented to lie in the plane of the membrane and these must make a greater contribution to the resistance than a randomly oriented collection of the same size and shape distribution. Microscopic examination also showed that the crystal size increased with wax concentration; at 1% wax the crystals were so small that their shape could barely be ascertained at 100 power magnification, whereas at 50% wax, the field was filled with crystals, most of which were of the order of or greater than the filter thickness. (This tendency would be expected if the number of crystallization nuclei did not increase in proportion with the wax content.) The orientation effect which must follow would explain the large increase in tortuosity between 10 and 50% wax.

In summary, solids may contribute to diffusion resistance in greater proportion than their volume fraction if they are anisometric and even more so if they are appropriately oriented in a barrier. Both of these factors will depend on the crystal habit and nucleation rate in the membrane medium as well as on the geometry of the phase in which the crystals grow.

Similar data on mixtures of cottonseed oil and cholesteryl palmitate are presented in Table VI. The 3.3% mixture is a fluid suspension of very thin platelets; the 33 and 58% mixtures are soft, white greases. The pure cholesteryl

palmitate is a hard, waxy solid. Comparing these results with the data of Table V, it is evident that the cholesterol ester is a much more effective inhibitor of diffusion than beeswax. This may be due in part to the platelet crystal habit of the former, but it seems unlikely that this property alone could account for the extremely high tortuosity (190) obtained with the 33% mixture nor would it account for the peculiar minimum in the permeability at about this composition.

A more plausible alternative explanation is that some of the components of the cottonseed oil enter into a multicomponent liquid crystalline phase with the cholesteryl palmitate increasing the volume fraction of the less permeable material.

Summary

The study of water permeability of skin model membranes consisting of lipid-impregnated Millipore filters has been continued. The theory of the measurements is discussed taking account of resistances due to evaporation and air diffusion.

The permeability of Millipore filters impregnated with three mineral oils increased with increasing oil fluidity but less rapidly than in direct proportion. No viscosity effect was detected in a corresponding series of silicone oils.

Small amount of insoluble, solid beeswax markedly reduced the permeability of cottonseed oil. The effect of cholesteryl palmitate in cottonseed oil was even more pronounced.

Table I

LIST OF SYMBOLS

α	Condensation coefficient.
b	Diffusion path length of resistance equivalent to evaporation resistance at liquid-vapor interface.
c	Concentration.
c_o	Equilibrium vapor concentration.
D	Diffusion coefficient.
E	Voltage.
F	Experimentally determined rate of evaporation.
F_o	Theoretical value of F from kinetic theory of ideal gases.
J	Flux.
k	Henry's law constant.
L	Air diffusion path length.
L_e	Air diffusion path length corresponding to a given resistance.
m	Mass of molecule.
p	Partial pressure of vapor.
p_o	Equilibrium vapor pressure.
P	Total pressure.
Q	$(kT/2\pi m)^{\frac{1}{2}}$.
r	Radius of diffusing particle.
R	Resistance.
R_m	Membrane resistance.
R_t	Total resistance.
S	Solubility.
v	Viscosity.
Δx	Thickness of barrier element.

Table II

AIR AND MONOFILM RESISTANCE MEASUREMENTS

Experiment No.	Conditions	Flux, J mg./cm.^{2}/hr.	Equivalent Air Path Length, L_e cm.	Path Length Increase ΔL, Due to Air or Monofilm cm.	Resistance of Air or Monofilm sec./cm.
1	Cup brim full	35.6	0.60	0.6[a]	2.3
2	Cup brim full	37.6	0.56	0.6[a]	2.3
3	Cup filled to *ca.* 1 cm. below brim	13.5	1.57	0.6[a]	2.3
4	MP Filter. Filled to *ca.* 1 cm. below brim	10.9	1.94	0.9[a]	3.5
5	MP Filter. Filled to *ca.* 0.2 cm. below brim	16.6	1.28	1.1[a]	4.3
6	MP Filter. Filled to top, filter wet	23.1	0.92	0.9[a]	3.9
7	MP Filter. Filled to 0.98 cm. below brim	9.7	2.19	1.21[a]	4.73
8	MP Filter. Filled to 0.47 cm. below brim	12.9	1.64	1.17[a]	4.57
9	Cup brim full	30.5	0.70	0.7[a]	-
10	Same as #9 after spreading film of hexadecanol	22.0	0.96	0.26[b]	1.02[b]
11	Cup filled to *ca.* 0.1 cm. below brim	25.8	0.82	0.7[a]	-
12	Same as #11 after spreading film of hexadecanol	18.9	1.12	0.30[b]	1.17[b]

[a] Due to air path outside cup.

[b] Due to monofilm only.

Table III

FLUX OF WATER VAPOR THROUGH OIL IMPREGNATED MILLIPORE FILTERS

Experiment No.	Oil	Mg. Oil on Filter[a]	Flux, J mg./cm.^{2}/hr.
1	Cottonseed	107	0.869
2	"	104	0.850 - Mean 0.86
3	"	103	0.857
4	Mineral, 0.33 poise/25°C.	100	0.255
5	Mineral, 0.33 poise/25°C.	100	0.265 - Mean 0.27
6	Mineral, 0.33 poise/25°C.	102	0.283
7	Mineral, 2.49 poise/25°C.	102	0.172
8	Mineral, 2.49 poise/25°C.	102	0.181 - Mean 0.18
9	Mineral, 2.49 poise/25°C.	102	0.174
10	Mineral, 224 poise/25°C.	100	0.083
11	Mineral, 224 poise/25°C.	99	0.094 - Mean 0.090
12	Mineral, 224 poise/25°C.	96	0.089
13	Silicone, 0.95 poise/25°C.	119	1.46
14	Silicone, 0.95 poise/25°C.	118	1.48 - Mean 1.47
15	Silicone, 9.85 poise/25°C.	120	1.43
16	Silicone, 9.85 poise/25°C.	122	1.46 - Mean 1.44
17	Silicone, 122 poise/25°C.	116	1.45
18	Silicone, 122 poise/25°C.	116	1.50 - Mean 1.47

[a]Disc, 47 mm. diameter.

Table IV

WATER VAPOR DIFFUSION RESISTANCE OF OIL
IMPREGNATED MILLIPORE FILTERS: VISCOSITY EFFECT

Oil Type	Visc., 25°C. Poise	Flux, J mg./cm.^{2}/sec.	Total Resistance, R_t sec./cm.	Membrane Resistance, R_m sec./cm.	Visc./R_m
Triglyceride (Cottonseed)	0.5	0.86	96.3	91.2	0.005
Mineral	0.33	0.27	307	302	0.0011
"	2.49	0.18	460	455	0.0055
"	224	0.09	920	915	0.245
Silicone	0.95	1.48	56.3	51.2	0.019
"	9.85	1.44	57.5	52.4	0.188
"	122	1.47	56.3	51.2	2.38

Table V

WATER VAPOR DIFFUSION THROUGH MILLIPORE FILTERS IMPREGNATED WITH COTTONSEED OIL - BEESWAX MIXTURES

% Beeswax	Flux, J mg./cm.^{2}/hr.[a]	Membrane Resistance, R_m sec./cm.	J (Oil) x Vol. Fract. mg./cm.^{2}/hr.	Tortuosity Factor
0	0.86	91.2	0.86	(1.00)
1	0.81	97.1	0.85	1.05
10	0.50	161	0.77	1.56
50	0.10	823	0.43	4.30
100	0.05	1650	-	-

[a]Means of duplicate measurements.

Table VI

WATER VAPOR DIFFUSION THROUGH MILLIPORE FILTERS IMPREGNATED WITH COTTONSEED OIL - CHOLESTERYL PALMITATE MIXTURES

% Chol. Palmitate	Flux, J mg./cm.^{2}/hr.	Membrane Resistance, R_m sec./cm.	J (Oil) x Vol. Fract.	Tortuosity Factor
0	0.86	91.2	0.86	(1.00)
3.3	0.50[a]	161	0.83	1.66
33	0.003[b]	3×10^4	0.58	190
58	0.02[b]	4×10^3	0.36	18
100	0.22[a]	370	-	-

[a]Means of duplicate measurements.

[b]Means of triplicate measurements.

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10. GENERAL DISCUSSION AND SUMMARY

A technique has been introduced which enables one to measure the skin barrier penetration by any material which has a significant vapor pressure at room temperature. The technique involves the use of ammonia-separated epidermal membranes which can be stored in the dried state for considerable periods of time and still retain their permeability characteristics. This enables one to set up a continuing series of experiments from a single pool of tissue.

The value of such a technique has been demonstrated in a series of studies on vapor diffusion of amines and alcohols. Alkyl amines penetrate skin much more readily than alcohols. With higher members (C_3 and up) of both classes of compounds, there is a general tendency for an increase in the permeability coefficients with increasing chain length. For straight chain members of a series with a chain length C_4 through C_7 (and possibly beyond), the logarithm of the permeability coefficient increases linearly with increasing number of carbon atoms. Branched-chain alkyl amine and alcohol isomers exhibit significantly lower permeability coefficients than their straight-chain counterparts.

Of further interest are the observations 1) that amine penetration data obtained using dry epidermis show good correspondence with the data reported earlier from these

laboratories for penetration of alkyl amines through whole skin in an aqueous system, and 2) that the barrier to water vapor penetration of epidermal membranes is to a large extent destroyed by amine vapor penetration. In earlier work done in these laboratories, it was found that the barrier of whole skin was damaged by passage of alkyl amines in aqueous solution.

A newly-designed apparatus and a new technique for the study of membrane penetration are described. The system is suitable both for free diffusion studies and for electrodialysis studies of chemical penetration of membranes. While whole skin can be used in this apparatus, we have found it more convenient to employ the ammonia-separated epidermal membranes referred to above.

In our investigations, we have confirmed the earlier report from our laboratories that there is virtually no penetration of an alkyl amine or an amino acid (glutamate) in the pH region where they possess a net charge of either sign. Under an applied potential at low currents (2 - 3 milliamperes), however, the positively charged species of ethoxyethylamine, and the negatively charged species of D- or L-glutamic acid can be electrodialyzed through epidermal membranes. There is no "lag time" observed in the process, and the compounds migrate at a constant rate when a constant current is maintained. Under our conditions, the positively charged ethoxyethylammonium ion shows a significantly higher electromigration rate than does the negatively charged glutamate ion.

Further studies will be directed toward elucidation of the factors which affect the electrodiffusion process. We believe that these investigations will give us further information on the nature of the skin barrier, as well as a means of assaying materials for their skin-penetrating propensities.

A procedure for the separation of the "barrier lipid fraction" (BLF) into major lipid components by silicic acid chromatography has been described. As judged by the order of elution, about 10% of the total BLF consists of waxes and sterol esters; about 30% of sterols and 60% consists of as yet unidentified polar lipids. The effectiveness of these fractions in providing water barrier to skin is being evaluated.

A series of acid hydrolyses, using 3, 4 and 6 N HCl, was conducted on stratum corneum cells of neonatal rats for time periods varying up to 24 hours and hexosamine release from the corneum was assayed. Maximum hexosamine was obtained within six hours with 3 N HCl and within one hour with 6 N HCl. The hexosamine values for the cells ranged from 0.15% to 0.18%.

Our studies of the essential fatty acid (EFA)-deficient state have been extended. In a previous report we discussed strengthening of the skin barrier in EFA-deficient animals using dietary safflower oil, a rich source of linoleate. In the current study, the effects of supplementation with linolenic acid, a trienoic fatty acid, as well as with a

source of linoleate, have been measured. Linolenic acid supplementation resulted in considerable improvement in the growth of EFA-deficient rats, but effected little, if any, decrease in skin permeability, as judged by electrical conductivity and water diffusion rate measurements. Linoleate supplementation resulted in a remission of dermal symptoms with a pronounced decrease in skin permeability, in addition to improvement in the growth rate. Even at the lowest level of linoleate supplementation, there was a final weight gain almost twice that obtained with linolenic acid (using 7 mg. linoleate vs. 35 - 100 mg. linolenic acid per rat per day).

The minimum level of linoleate adequate for full restoration and maintenance of skin barrier properties is surprisingly low; 7 mg. per rat per day seems to be a threshold level.

In the course of determining the fatty acid compositions of the epidermal lipid classes previously reported, it became apparent that current methods for fractionating fatty acids were not adequate. We have developed a new procedure for quantitatively separating the saturated fatty acids from a mixture containing unsaturated acids. This technique, which involves bromination on a thin layer chromatography plate, is simple and rapid and provides samples suitable for gas chromatographic analysis.

The close correspondence generally observed between the electrical conductivity of animal skin and its water

permeability has been made the basis for two *in vivo* studies on the skin of humans.

Conductivity measurements of 34 anatomical sites indicated that the electrical conductivity of skin of men may be related to the sweat gland density. The skin conductivity values observed at the sites with high sweat gland density were much higher than those found in sites with relatively few sweat glands. Of significance here is the similarity in conductivity values between human skin with low sweat gland density and those found on rodent skin which of course contains no sweat glands.

In an application of Szakall's method for successive removal of the corneum cell layers with cellophane tape, the skin conductivity changes showed strong similarity to the water permeability changes reported by Blank. Our values for skin conductivity and Blank's for water diffusion rate remained fairly constant during a number of strippings, and then increased sharply. These findings are in disagreement with those of investigators who have reported regular increases in skin permeability with successive removal of the outer layers of the corneum.

The water permeability of various lipoidal substances and mixtures has been studied by measurement of rates of vapor transmission through impregnated millipore filters corrected

for resistance due to air diffusion gaps.

Permeability decreased with increasing viscosity in a series of three mineral oils, but in a corresponding series of silicone oils there was no detectable viscosity effect. The permeability of a liquid triglyceride mixture (cottonseed oil) was markedly reduced by addition of beeswax and even more so by the addition of cholesteryl palmitate both of which are insoluble in the liquid oil. The effect on permeability of solids and of liquid crystals which may occur in skin lipids is discussed. These measurements are being extended to other lipids which are found in the epidermis.